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It contains the 24 papers presented during Sessions 1–4 of the Conference. Papers presented during Sessions 5–8 will be published in a second "Special Issue ILC2018" in the near future.

Editors

Management Committee

Editorial Board

Principal Contacts

Rainer Schultze-Kraft
International Center for Tropical Agriculture (CIAT)
Colombia
Phone: +57 2 4450100 Ext. 3036
Email: CIAT-TGFT-Journal@cgiar.org

Technical Support
José Luis Urrea Benítez
International Center for Tropical Agriculture (CIAT)
Colombia
Phone: +57 2 4450100 Ext. 3354
Email: CIAT-TGFT-Journal@cgiar.org

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Preamble

Leucaena is widely recognized as the most sustainable, and valuable multipurpose tree legume in the tropics. It is a productive and profitable source of protein for ruminant production. Its other uses include: land regeneration; carbon sequestration and methane reduction; and biomass for paper pulp and electricity generation. Over the past three decades, scientists and farmers have greatly increased their knowledge of this plant, resulting in new plantings that have increased almost exponentially over time. As a consequence, there is demand for improved knowledge of the latest varieties, recommended management practices and feeding systems.

This Issue of the Journal contains papers presented at a very successful International Leucaena Conference (ILC2018) including a pre-conference field tour, organized by The University of Queensland, staged from 29 October to 3 November 2018. The last dedicated conference on leucaena was held in Vietnam in 1997.

Approximately 120 conference delegates from 12 countries, comprising researchers, extension officers, consultants, producers and students, shared their research knowledge and practical experiences regarding leucaena. Many excellent speakers exchanged information regarding how to plant, manage and use leucaena around the world. Engagement and networking ensured there was enthusiastic and fruitful discussion on future priorities and collaborative opportunities.

Professor James Brewbaker

The Conference especially honored Professor James Brewbaker from the University of Hawaii, for his lifelong contribution to the understanding of the genetics and breeding of the *Leucaena* genus, to teaching and research supervision of students from around the world and for his support of ILC2018.

We acknowledge the help of many people, including members of the ILC2018 Organizing and Steering Committees and the Editorial panel who performed an important role in ensuring that submitted papers were of an acceptable standard. We especially thank the Editors of the Journal *Tropical Grasslands-Forrajes Tropicales* for their huge efforts in final reviewing, polishing and refining of manuscripts and ultimately preparing them for publication in the two issues of the Journal.

We also acknowledge Dr Nigel Tomkins and Mr Joe Rolfe for organizing the environmental and producer case studies sessions, respectively; and Meat and Livestock Australia, Australian Centre for International Agricultural Research and The University of Queensland for financial assistance.

Finally, special thanks are due to Dr Scott Dalzell for coordinating the pre-conference tour and the producers who kindly shared their experience and made their facilities and properties available for the very informative and successful tour. Participants received an excellent overview of how leucaena is used on properties in southeast Queensland, and engaged in extensive debate on a wide range of topics.

Conference delegates at The University of Queensland

We are proud of all that was achieved during the conference, and that it will be available to all in these special issues.

Assoc. Prof. Max Shelton and Dr Nahuel Pachas
ILC2018 Organizing Committee
The University of Queensland

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ILC2018 Plenary paper*

Leucaena cultivars – current releases and future opportunities

Cultivares de leucaena – estado actual y oportunidades futuras

SCOTT A. DALZELL

Leucaena Research and Consulting Pty Ltd, Port Macquarie, NSW, Australia.

Abstract

The *Leucaena* genus is made up of 24 different species (19 diploid and 5 tetraploid species). However, early use of the *Leucaena* genus in agricultural systems was based entirely upon a very narrow germplasm base. A single genotype of *Leucaena leucocephala* ssp. *leucocephala* ('common' leucaena) was spread pantropically from its center of origin in Mexico over 400 years ago. Genetic improvement of *Leucaena leucocephala* began in the 1950s, when vigorous 'giant' leucaena (*L. leucocephala* ssp. *glabrata*) was identified in Australia and Hawaii. Cultivars such as Hawaiian Giant K8, Peru and El Salvador were selected and promoted for grazing in Australia and multipurpose agroforestry uses throughout the tropics. Plant breeding for improved forage production resulted in the release of cv. Cunningham in 1976 in Australia. These cultivars of 'giant' *Leucaena leucocephala* displayed broad environmental adaptability, with the exception of poor tolerance of cold temperatures (and frost) and acid soils. The outbreak of the psyllid insect pest (*Heteropsylla cubana*) from Cuba during the 1980s devastated both 'common' and 'giant' leucaena all around the world. This challenge resulted in renewed interest in lesser-known *Leucaena* spp. that exhibited tolerance to the pest and in interspecific hybridization as a means of developing new cultivars. Some 'giant' leucaena lines exhibited excellent agronomic traits and a degree of tolerance to the psyllid pest and this resulted in the release of new cultivars in Australia (cvv. Tarramba and Wondergraze) and Hawaii (cv. LxL). Since the 1990s, plant breeding programs have sought to develop cultivars with greater psyllid tolerance using interspecific hybridization. This has resulted in the release of cv. 'KX2-Hawaii' for timber and forage production, and a backcrossed forage cultivar cv. Redlands (Australia). Both cultivars are based upon interspecific hybridization between *L. pallida* and *L. leucocephala* ssp. *glabrata*. Cold-temperature and acid-soil tolerance have been pursued in South American breeding programs based upon *L. diversifolia*, without commercial success. The development of sterile *Leucaena* spp. cultivars is currently underway to nullify the environmental weed potential of all current commercial cultivars. Tolerance to cold temperatures (*L. diversifolia, L. pallida, L. pulverulenta* and *L. trichandra*), frost (*L. greggii* and *L. retusa*) and psyllids (*L. collinsii*) exists within the *Leucaena* genus and may be exploited in future hybridization programs. New genetic analyses and molecular plant breeding techniques have the potential to facilitate further gene transfer between *Leucaena* spp. for the development of the next generation of multipurpose cultivars.

Keywords: Hybridization, plant breeding, psyllid resistance, tree legumes.

Resumen

El género *Leucaena* está compuesto por 24 especies diferentes (19 diploides y 5 tetraploides). Sin embargo, en su primera fase el uso del género *Leucaena* en sistemas agropecuarios se basó exclusivamente en una estrecha base de germoplasma. Un solo genotipo de *Leucaena leucocephala* ssp. *leucocephala* (leucaena 'común') fue el que que hace más de 400 años se dispersó pantropicalmente desde su centro de origen en México. El mejoramiento genético de *Leucaena leucocephala* comenzó en la década de 1950, cuando se identificó una vigorosa leucaena 'gigante' en Australia y Hawái, *L. leucocephala* ssp. *glabrata*. Cultivares como Hawaiian Giant K8, Peru y El Salvador fueron seleccionados y promovidos para pastoreo en Australia y usos agroforestales múltiples en todo el trópico. Un programa de fitomejoramiento buscando mayor rendimiento de forraje resultó en la liberación del cv. Cunningham en 1976 en

Correspondence: S.A. Dalzell, Leucaena Research and Consulting Pty Ltd, 13 Wonga Crescent, Port Macquarie, NSW 2444, Australia. Email: dalzellagadvisory@gmail.com

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Australia. Los cultivares del tipo 'gigante' de *Leucaena leucocephala* mostraron una amplia adaptabilidad a las condiciones ambientales, con excepción de tolerancia a temperaturas bajas (incluyendo heladas) y suelos ácidos. El brote del insecto plaga *Heteropsylla cubana* (Psyllidae) durante la década de 1980 tuvo un efecto devastador en las leucaenas 'común' y 'gigante' en todo el mundo. Este desafío dio lugar a un renovado interés en especies menos conocidas de *Leucaena* que mostraran tolerancia a la plaga, y en la hibridación interespecífica como medio para desarrollar nuevos cultivares. Algunas líneas de leucaena 'gigante' exhibieron excelentes características agronómicas y cierta tolerancia a la plaga de los psílidos, lo que dio lugar a la liberación de nuevos cultivares en Australia (cvv. Tarramba y Wondergraze) y Hawái (cv. LxL). Desde la década de 1990, programas de fitomejoramiento han buscado desarrollar cultivares con mayor tolerancia a los Psyllidae utilizando la hibridación interespecífica. Como resultado se liberó el cv. 'KX2-Hawaii' para la producción de madera y forraje, y cv. Redlands en Australia, un cultivar forrajero retrocruzado. Ambos cultivares están basados en la hibridación interespecífica entre *L. pallida* y *L. leucocephala* ssp. *glabrata*. En Sudamérica se llevaron a cabo proyectos de mejoramiento basados en *L. diversifolia* buscando tolerancia a temperaturas bajas y suelos ácidos, sin embargo sin éxito comercial. Proyectos actualmente en curso tienen como objetivo desarrollar cultivares de *Leucaena* spp. estériles para eliminar el potencial de maleza ambiental de los actuales cultivares comerciales. Dentro del género *Leucaena* sí existen características como tolerancia a temperaturas bajas (*L. diversifolia, L. pallida, L. pulverulenta* y *L. trichandra*), a heladas (*L. greggii* y *L. retusa*) y a los psílidos (*L. collinsii*) y se podrán explotar en futuros programas de hibridación. Las nuevas técnicas disponibles de análisis genético y reproducción molecular de plantas tienen el potencial de facilitar la transferencia de genes entre especies de *Leucaena* con el fin de desarrollar la próxima generación de cultivares multipropósito.

Palabras clave: Fitomejoramiento, *Heteropsylla cubana*, hibridación, leguminosas arbóreas, resistencia a plagas.

History

Utilization of multipurpose trees from the 24 species of the *Leucaena* genus (Abair et al. 2019) has been occurring for millenia in subsistence agricultural systems in seasonally dry forest areas throughout their native range extending from southern Texas, USA to northern Peru (Hughes 1998). In the 16th century, Spanish colonists in Central America recognized the potential of leucaena as an animal forage and began the spread of 'common' weedy leucaena (*L. leucocephala* ssp. *leucocephala*) throughout the tropics (Gray 1968; Brewbaker 2016). 'Common' leucaena is a small branchy tree with low biomass yield, poor form, early flowering and heavy seed production (Gray 1968). This remarkable plant has wide adaptability to a range of soil types and climatic conditions, where it has become established in disturbed environments (Campbell et al. 2019; Idol 2019). 'Common' leucaena has been utilized by subsistence smallholder farmers pantropically to produce fuelwood, timber, green manure, shade, animal forage and human food. It has also been trialled for use in commercial agriculture as a fodder for ruminant animals (Takahashi and Ripperton 1949; Kinch and Ripperton 1962).

During the 1950s agronomists and plant breeders in Australia and Hawaii began programs to identify and develop superior leucaena cultivars for adoption in commercial agricultural systems (Gray 1968; Brewbaker 2016). Seed of 'common' leucaena was collected from disparate areas and evaluated. It soon became apparent that 'common' leucaena lacked diversity in key agronomic characteristics (Gray 1968), indicating that this phenotype was genetically identical all around the world, having originated from a narrow genetic base. This was later confirmed by molecular genetic analysis (Sun 1992).

Advances in genetic improvement followed the identification and commercialization of 'giant' types of leucaena (*L. leucocephala* ssp. *glabrata*) in Hawaii (Hawaiian Giant K8 – 1975, K28, K29, K67 and K72; Brewbaker et al. 1972) and Australia (El Salvador – 1962, Peru – 1962, Tarramba – 1997; Gray 1968; Oram 1990). The 'giant' types had superior vigor/yield and less precocious seed production (Hutton and Gray 1959; Brewbaker et al. 1972; Brewbaker 1975). Tree form varied within the 'giant' types, with some accessions being arboreal (Hawaiian Giant K8, El Salvador and Tarramba), while others had a greater degree of basal branching (Peru). These early cultivars of 'giant' leucaena have been widely distributed around the world for use in tropical agroforestry systems. A comprehensive, authoritative review of the history of genetic improvement of the *Leucaena* genus has been compiled by Professor J.L. Brewbaker, University of Hawaii (UH) (Brewbaker 2016).

Plant breeding programs have combined the superior attributes of different accessions of 'giant' leucaena, with breeding objectives including: increased forage yield and branched tree form suitable for direct grazing; and more recently, tolerance of the psyllid insect.

Cultivar Cunningham (public domain cultivar in Australia) is an intraspecific hybrid based upon cv. Peru and was released as a forage type by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) within Australia in 1976 (Oram 1990). It was selected for superior forage yield and branched form (Hutton and Beattie 1976). Cunningham has been widely planted in Australia and around the world.

The emergence of a devastating pantropical psyllid insect pest (*Heteropsylla cubana*) during 1983–1990 (Bray 1994), triggered a second phase of genetic evaluation and cultivar development. A number of accessions of 'giant' leucaena had moderate tolerance to the psyllid. These formed the basis of the following cultivars:

- cv. LxL: A synthetic line of 6 intraspecific hybrid forage breeding lines released by the University of Hawaii (UH) in 1996 (Austin et al. 1998). Despite having superior forage yield (~15% heterosis), cv. LxL has had limited commercial utilization in the USA (Brewbaker 2016).
- cv. Tarramba (protected by PBR in Australia): A bred line (UH) from accession K636 collected from highlands in Coahuila, Mexico (Brewbaker 2016) and released in Australia in 1997 (Anonymous 1997). Key attributes of cv. Tarramba are: erect arboreal habit; excellent biomass and forage production; some cool-temperature tolerance; moderate tolerance of the psyllid insect pest; and reduced seed production. Cultivar Tarramba has been readily adopted in smallholder ruminant feeding systems in Indonesia (Kana Hau and Nulik 2019), where its erect stems are valued for fuelwood and construction timber.
- cv. Wondergraze (protected by PBR in Australia): Selfed progeny (S4) from an intraspecific cross between accession K584 and cv. Tarramba bred by UH and released in Australia in 2010 (Anonymous 2008). Key attributes of cv. Wondergraze are: moderate tolerance of the psyllid insect pest; good forage yield; branched tree form; and excellent seedling vigor.

Environmental limitations to 'giant' leucaena

The following environmental constraints restrict the productivity of 'giant' leucaena: defoliation by frost; poor growth under cool temperatures; and lack of tolerance of acid soils (Hutton 1983). While 'giant' leucaena can survive severe frost by regrowing from the root crown during spring (Felker et al. 1998), minor frost (0 to -3 °C) burns the leaves from plants and moderate frost (<-3 °C) kills stems to ground level (Dalzell et al. 1998a; Middleton and Clem 1998;). This restricts the ability of farmers in subtropical areas to utilize 'giant' leucaena forage during the winter protein feed gap, when it would be of tremendous benefit to livestock production. Growth of 'giant' leucaena slows significantly when average daily temperatures drop below 25 °C and average monthly minimum temperatures drop below 22 °C (Mullen et al. 2003c), restricting forage production during spring and autumn in subtropical areas and year-round production in the elevated tropics. 'Giant' leucaena thrives on neutral-alkaline calcareous soils. It grows poorly on acid soils (pH water 1:5 <5.2) due to calcium and phosphorus deficiency and aluminum toxicity adversely impacting root growth, rhizobium nodulation and nitrogen fixation (Hutton 1983). There are large tracts of acid soils in tropical areas that would otherwise be suitable for 'giant' leucaena development.

Interspecific hybridization

Many of the lesser-known *Leucaena* spp. have agronomic traits that address the limitations to adaptation of *L. leucocephala* ssp. *glabrata*, including: psyllid resistance (*L. collinsii*, *L. esculenta* and *L. pallida*) (Mullen et al. 2003b); cold tolerance (*L. diversifolia*, *L. pallida* and *L. trichandra*) (Mullen et al. 2003c); and frost tolerance (*L. greggii*, *L. pulverulenta* and *L. retusa*) (Hughes 1998). However, these species cannot be commercialized directly in agroforestry systems because they have other serious limitations to utility such as low biomass/forage yield (*L. greggii* and *L. retusa*) (Mullen et al. 2003a), poor forage quality (*L. diversifolia*, *L. esculenta*, *L. greggii*, *L. pallida*, *L. pulverulenta* and *L. trichandra*) (Dalzell et al. 1998b; Jones et al. 1998) and potentially a lack of longevity or tolerance of regular defoliation (*L. esculenta*, *L. greggii*, *L. pallida* and *L. retusa*) (Mullen et al. 2003a).

A high degree of interspecific cross compatibility has been identified within the *Leucaena* genus (Gonzalez et al. 1967; Sorensson and Brewbaker 1994). Interspecific hybridization enables plant breeders to combine superior traits from different species to form the basis of populations for further selection and genetic improvement. Hybridization programs have been undertaken to develop new cultivars of *Leucaena* with the following characteristics:

Low mimosine forage

Variability in concentration of the toxic amino acid mimosine in foliage exists within the *Leucaena* genus. Species with lower concentrations of mimosine include: *L. pulverulenta* (Gonzalez et al. 1967; Brewbaker et al.

1972; Bray et al. 1984); and *L. collinsii*, *L. diversifolia*, *L. shannonii* and *L. trichandra* (Brewbaker and Kaye 1981; Saunders et al. 1987). Hybridization to produce low-mimosine forage cultivars for feeding to mono-gastric animals has been attempted. Hybrid lines of *L. pulverulenta* × *L. leucocephala* ssp. *glabrata* were developed with low mimosine concentration in Australia; however, the programs were unsuccessful as low-mimosine breeding lines (~25% reduction in mimosine) had significantly lower forage yields than existing commercial cultivars (Bray et al. 1984).

Acid soil tolerance

In general, little specific tolerance to acid soils was identified within the *Leucaena* genus by a comprehensive environmental adaptation study (Mullen et al. 2003c). However, acid soil tolerance has been reported within accessions of *L. diversifolia* and *L. trichandra* (Hutton 1983). Hybrid breeding programs (× *L. leucocephala* ssp. *glabrata*) in South America (CIAT/EMBRAPA) and Southeast Asia (MARDI) have been undertaken to develop psyllid-tolerant and acid soil-tolerant forage cultivars (Wong et al. 1998). Two hybrid cultivars were released in Malaysia in 1998 (Aminah and Wong 2004), cv. Bharu (*L. trichandra* × *L. leucocephala* ssp. *glabrata* breeding line 40-1-18) and cv. Rendang (*L. diversifolia* × *L. leucocephala* ssp. *glabrata* breeding line 62-6-8). The commercial success of these cultivars is unknown and wider assessment of their agronomic performance and forage quality (palatability and digestibility) is required. Concentrations of condensed tannins in both cultivars have been reported to be high and to adversely impact rumen function (Khamseekhiew et al. 2000; Kok et al. 2013; Saminathan et al. 2015, 2017). These hybrid cultivars need to be compared with alternative multi-purpose shrub legumes with known acid soil tolerance, e.g. *Calliandra calothyrsus*, *Cratylia argentea* and *Flemingia macrophylla*.

Psyllid resistance

Hybrid cultivars have been developed from *L. pallida* × *L. leucocephala* ssp. *glabrata* (designated KX2 hybrids) for forage and biomass/timber. These hybrids have shown high yield with broad environmental adaptation (Mullen et al. 2003c), psyllid resistance (Mullen et al. 2003b), cool tolerance (Austin et al. 1997; Mullen et al. 2003c) and intermediate forage quality (Dalzell et al. 1998b).

- Cultivar KX2-Hawaii was bred by UH and was released in 2007 (Brewbaker 2008). This cultivar was developed by 6 cycles of recurrent selection from advanced generations of the original F1 hybrid *L. pallida* K376 × *L. leucocephala* ssp. *glabrata* K8. It was selected under regular cutting/coppicing for psyllid resistance, forage/biomass yield and self-sterility. To date, there has been limited commercial utilization of cv. KX2-Hawaii.
- Cultivar Redlands (protected by PBR in Australia) was bred by the University of Queensland (Anonymous 2015) and was released in 2017. This hybrid cultivar was developed using 5 elite KX2 F1 hybrids bred by UH. These parents were open-pollinated (panmixia) and F2 seed planted for intense selection (5–10% retention) under the criteria of psyllid resistance, yield, tree form (high degree of basal branching) and self-sterility. After another cycle of recurrent mass selection, elite F3 trees were backcrossed (BC) (hand-pollinated) to *L. leucocephala* ssp. *glabrata* cv. Wondergraze. Elite psyllid-resistant BC progeny were backcrossed again to produce breeding lines that were effectively 87.5% cv. Wondergraze and 12.5% *L. pallida*. The best BC2 breeding lines were then self-pollinated 3 times. Selfed breeding lines were assessed for in vitro forage quality (digestibility plus crude protein and condensed tannin concentrations) and their palatability determined under direct grazing. Cattle had a preference for cvv. Cunningham and Wondergraze plots ahead of cv. Redlands, but cv. Redlands was readily eaten (Shelton et al. 2019). A trial comparing hedgerow pastures of cvv. Wondergraze and Redlands and measuring cattle liveweight gain is currently underway in north Queensland (Lemin et al. 2019). Cultivar Redlands is recommended for humid (average annual rainfall >800 mm) psyllid-prone areas.

Cold tolerance

Hybrids based upon *L. diversifolia* (KX3) and *L. pallida* (KX2) with *L. leucocephala* ssp. *glabrata* have been developed by UH and distributed for evaluation throughout the tropics (Brewbaker 2016). These hybrids are vigorous, psyllid-resistant/tolerant and have superior growth under cool temperatures during autumn and spring in the subtropics (Middleton and Clem 1998; Mullen et al. 2003a, 2003c) and year-round in the elevated tropics (Austin et al. 1997). The forage quality of these hybrids requires careful evaluation, as it is likely to be lower than 'giant' leucaena owing to higher concentrations of condensed tannins inherited from *L. diversifolia* and *L. pallida* (Austin et al. 1997; Dalzell et al. 1998b). With the exception of cv. KX2-Hawaii, no cultivars from this breeding program have been commercialized. KX3 hybrids have been developed and evaluated in southern

Brazil (Austin et al. 1998) and Argentina (Goldfarb and Casco 1998) for frost and cold tolerance; however, no known commercial cultivars have been released from these programs.

Wood/biomass/pulp production

Fast-growing *Leucaena* spp. hybrids have great potential for high-value timber, biomass (bioenergy) and paper pulp production (Brewbaker 2016). Cultivar KX4-Hawaii is a male-sterile triploid hybrid between *L. leucocephala* ssp. *glabrata* K636 and *L. esculenta* K838 developed by UH (Brewbaker 2013). This hybrid is vegetatively propagated, psyllid-tolerant, arboreal, vigorous and cool-tolerant. Significant areas (>18,000 ha) of 'giant' leucaena in Gujarat, Maharashtra and Madhya States in India are managed for wood production to supply paper pulp mills (Khanna et al. 2019). Genetic improvement of 'giant' leucaena germplasm has been undertaken through intense selection and mutagenesis to improve biomass yield. A triploid *L. collinsii* × *L. leucocephala* ssp. *glabrata* hybrid has been developed by JK Paper Ltd (Khanna et al. 2019) and is currently being vegetatively propagated for evaluation of biomass yield and paper pulp characteristics. This hybrid also has potential as a forage plant and requires wider evaluation for environmental adaptation, forage production and animal feeding.

Sterility

'Common' and 'giant' leucaena have the potential to become environmental weeds of disturbed ruderal habitats in the absence of grazing animals (Campbell et al. 2019; Idol 2019). Breeding programs within Australia are currently developing sterile cultivars for use in extensive grazing systems in jurisdictions where the promotion of 'giant' leucaena is not sanctioned. Strategies are focussing on developing sterility (male or female) in commercial cultivars via mutagenesis (McMillan et al. 2019) or gene editing to prevent flowering (Real et al. 2019). Interspecific hybridization to develop sterile triploids is also being explored (Real et al. 2019). In addition to reducing or eliminating the weed potential of *Leucaena* spp. cultivars, sterility may confer a significant yield (forage or biomass) advantage as plant resources are not diverted from vegetative growth to seed production.

Future directions for cultivar development

Superior accessions of lesser-known *Leucaena* spp. (Table 1) have been identified in extensive germplasm evaluation trials (Mullen et al. 2003a; 2003b; 2003c). These could be utilized to develop new interspecific hybrids to overcome the lack of cold, frost and acid soil tolerance in current commercial cultivars. Other accessions within these lesser-known taxa are held within international germplasm collections and require further agronomic evaluation (consult the World Leucaena Catalogue; Bray et al. 1997).

As understanding of the genetic base of the *Leucaena* genus improves, new tools have become available for plant breeding. Phylogenetic studies of the evolutionary history of the *Leucaena* genus have identified the parents of the 5 allotetraploid species (Govindarajulu et al. 2011a) and enabled the definition and elucidation of relationships between the 19 diploid species (Govindarajulu et al. 2011b; Abair et al. 2019). Sequencing of the *L. trichandra* genome has been completed and will enable genetic markers to be identified for traits of interest in breeding programs (Abair et al. 2019). Application of molecular marker-assisted selection should accelerate rates of genetic gain in traditional and molecular plant breeding programs.

Chromosome/ploidy doubling has been successfully undertaken in a number of *Leucaena* spp. (Shi 2003). Diploid species could be doubled, which may enhance cross-compatibility for desired interspecific hybrids. *Leucaena collinsii* (2n) is of particular interest as a forage plant as it is psyllid-resistant (Mullen et al. 2003b), has moderate forage yield (Mullen et al. 2003a; 2003c), excellent in vitro forage quality (Dalzell et al. 1998b) and has proved productive under cattle grazing (Jones et al. 1998). Producing and evaluating artificial tetraploid *L. collinsii* lines could deliver valuable new forage cultivars. Similarly, halving ploidy levels of the tetraploid *Leucaena* spp. would generate diploid (2n) lines that could be used to develop sterile triploid cultivars. Gametophytic self-incompatibility systems could be used to produce F1 interspecific hybrid seed (Brewbaker 2016).

New genetic technologies have potential to modify the *Leucaena* genome, including transgenic improvement, e.g. suppressing mimosine synthesis by the transfer of a gene from *Rhizobium* sp. into *L. leucocephala* ssp. *glabrata* K636 via agrobacterium (Jube and Borthakur 2010), or gene deletion using CRISPR technology (Real et al. 2019). Mutagenesis has been used to alter the genome of *L. leucocephala* ssp. *glabrata* to increase plant yield (Khanna et al. 2019) or induce sterility (McMillan et al. 2019).

Modern vegetative propagation techniques can be used for embryo rescue of F1 interspecific hybrid seeds that are prone to abort and to mass produce elite sterile germplasm for commercial application.

Table 1. Superior accessions of key *Leucaena* spp. for use in future hybridization programs [adapted from Mullen et al. (2003a; 2003b; 2003c)].

Breeding objective	Constraint	Taxon	Accession
Forage	Cold tolerance	*L. diversifolia*	K778, K784, K806, OFI104/94, CPI33820
		L. pallida	K748, K802, K376, CQ3439
		L. trichandra	OFI53/88, OFI35/88
	Frost tolerance	*L. retusa*	-[1]
	Sterility (2n parent)	*L. collinsii*	OFI51/88, OFI52/88
		L. magnifica	OFI1984, OFI58/88
Timber/biomass/ paper pulp/shade	Cold tolerance	*L. diversifolia*	K778, K784, K806, OFI104/94, CPI33820
		L. pallida	K748, K802, K376, CQ3439
		L. trichandra	OFI53/88, OFI35/88
	Frost tolerance	*L. pulverulenta*	-[1]
		L. greggii	-[1]
		L. retusa	-[1]
	Sterility (2n parent)	*L. cruziana*	OFI51/87
		L. esculenta	-[1]
		L. magnifica	OFI1984, OFI58/88
		L. macrophylla ssp. *istmensis*	OFI47/85
		L. macrophylla ssp. *macrophylla*	OFI55/88
		L. multicapitula	OFI81/87
		L. pulverulenta	-[1]
		L. salvadorensis	-[1]
		L. trichandra	OFI53/88, OFI35/88

[1]Superior accessions not identified – evaluation of a diverse array of accessions required.

Challenges and opportunities for future cultivar development

Many of the lesser-known *Leucaena* taxa have been identified only within the last 30 years and are represented by few accessions/provenances, e.g. *L. confertiflora*, *L. cuspidata*, *L. involucrata*, *L. lempirana*, *L. magnifica*, *L. matudae* and *L. pueblana*, from limited geographical areas in international germplasm collections (Hughes 1998; Brewbaker 2016). Further germplasm collection, conservation, multiplication and evaluation of these taxa are required. In addition, recent advances in *Leucaena* taxonomy (Abair et al. 2019) and the use of molecular markers will enable the accurate description of germplasm currently held (often misidentified and/or duplicated) in international collections and facilitate a much-needed update of the World Leucaena Catalogue (Bray et al. 1997). The World Leucaena Catalogue could be promoted as a 'source of truth' for the identification of *Leucaena* spp. accessions exchanged for use in future breeding programs. Germplasm collections are expensive to maintain, as seed needs to be refreshed and multiplied. Seed of some species, e.g. *L. esculenta*, appears to have a shorter lifespan under long-term storage.

A number of important practicalities must be considered when formulating *Leucaena* spp. breeding programs, including: focussing on forage quality for multipurpose tree legumes to ensure the forage produced fattens animals; long-term field testing of interspecific hybrids or elite lesser-known species to ensure longevity under frequent cutting or heavy grazing; determining the promiscuity of new cultivars for *Rhizobium* spp. to facilitate effective nodulation and adequate rates of biological nitrogen fixation (Mullen et al. 1998); estimating the cost of producing propagules (seed vs. vegetative planting material) at a commercial scale suited for adoption in target farming systems; and understanding the environmental requirements and establishment practices (seed vs. vegetative planting material) required for rapid widespread adoption of new cultivars.

Finally, a key challenge to breeding *Leucaena* is the long time-frame (>10 years) and significant resources (financial and human) required to develop new cultivars. Collaboration between international breeding programs would make the most of these limited resources. Such collaboration may include: the exchange of successful breeding technologies/techniques and elite germplasm; and undertaking coordinated G × E trials of advanced breeding lines and emerging cultivars. The spirit of such collaboration has been epitomized by Professor James L. Brewbaker (University of Hawaii), who for over 50 years has generously shared his vast knowledge of *Leucaena* spp. collection, genetics and breeding plus elite germplasm with plant breeders around the world.

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(Note of the editors: All hyperlinks were verified 17 April 2019.)

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The evolutionary history of *Leucaena*: Recent research, new genomic resources and future directions

La historia evolucionaria de Leucaena: *Investigaciones recientes, recursos genómicos nuevos y futuras perspectivas*

ALEXANDER ABAIR[1], COLIN E. HUGHES[2] AND C. DONOVAN BAILEY[1]

[1]*Department of Biology, New Mexico State University, Las Cruces, NM, USA.* bio.nmsu.edu
[2]*Department of Systematic & Evolutionary Botany, University of Zurich, Zürich, Switzerland.* systbot.uzh.ch

Abstract

Ancestral genome duplication, genomic diploidization, allopatric diploid speciation and recent allotetraploidy (hybrid tetraploid formation) have all contributed to the complex evolutionary history of the genus *Leucaena* Benth. (Leguminosae: Caesalpinioideae: mimosoid clade). This complexity makes *Leucaena* an exemplary group to investigate the impacts of these diverse mechanisms on plant speciation across time and space. Furthermore, this complex evolutionary history offers unique opportunities and challenges for translational applied research to improve the use of *Leucaena* in agroforestry, livestock production, soil stabilization and enrichment and biofuels. Here we review and synthesize historical and recent research on the evolutionary history of *Leucaena* and highlight the availability of new genomic data resources and tools.

Keywords: Hybridization, Leguminosae, phylogenetics, polyploidy, speciation, systematics.

Resumen

La duplicación del genoma ancestral, la diploidización genómica, la especiación diploide alopátrica y la alotetraploidía reciente (formación de tetraploides híbridos) han contribuido a la compleja historia evolutiva del género *Leucaena* Benth. (Leguminosae: Caesalpinioideae: clado mimosoide). Esta complejidad hace de *Leucaena* un grupo ejemplar para investigar los impactos de estos diversos mecanismos en la especiación de plantas a través del tiempo y el espacio. Además, esta compleja historia evolutiva ofrece oportunidades y desafíos únicos para la investigación translacional aplicada con el objetivo de mejorar el uso de *Leucaena* en agroforestería, producción ganadera, estabilización y enriquecimiento del suelo, y biocombustibles. En este trabajo revisamos y sintetizamos la investigación histórica y reciente sobre la historia evolutiva de *Leucaena* y destacamos la disponibilidad de nuevos recursos de datos genómicos y herramientas para procesarlos.

Palabras clave: Especiación, filogenética, hibridación, Leguminosae, polyploidía, sistemática botánica.

Introduction

The neotropical legume genus *Leucaena* comprises 24 species, with a native range spanning the southern USA to northern Peru. *Leucaena* are mostly small trees (occasionally shrubs) with bipinnately compound leaves, a lack of stem or leaf armament, extrafloral nectaries, globose to subglobose inflorescences of many small flowers and elongate, flattened, dehiscent pods. Numerous human uses for *Leucaena* have contributed to a long history of use in Mesoamerica for food, shade, firewood and even spiritual medicine. Archaeological evidence from seed remains in caves dates the use of *Leucaena* seeds as a minor food source by Mixtec and Nahuatl people to at least 6,000 years ago

Correspondence: Alexander Abair, Department of Biology, New Mexico State University, Las Cruces, NM 88003-8001, USA. Email: aabair@nmsu.edu

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(Zárate 2000) and seeds of 13 species have been recorded to be consumed in modern times in south-central Mexico (Hughes et al. 2007), where they are referred to as 'guaje' [goo-ah'-hay].

Research on *Leucaena* has focused on the archaeological history of plant use, patterns of evolutionary diversification among species, impacts of human use on diversification, as well as many applied research questions associated with multipurpose use in subsistence farming, modern agriculture and range management systems. The number of distinct species within the *Leucaena* genus has been investigated through reciprocal illumination of morphological and molecular evidence that currently supports the recognition of 19 diploid and 5 allotetraploid species (Hughes 1998a; Govindarajulu et al. 2011a). Studies into the underlying patterns of divergence and reticulation among species have involved phylogenetic, geographic and crossability projects. The morphological and ecophysiological diversity within the genus combined with high crossability among species provide ample opportunities for genetic improvement via traditional breeding approaches, and notably via interspecific artificial hybridization to develop genetically improved seed lines (Brewbaker et al. 1989; Brewbaker and Sorensson 1990). Additionally, newly generated genomic data, tools and resources are helping to advance our understanding of species relationships and will be critical for underpinning future basic and applied research on this interesting and economically important genus.

Here we provide an overview of species diversity and the evolutionary history of *Leucaena*, focusing especially on studies over the last 30 years. These studies have revealed a complex history, which includes paleopolyploidy, genomic diploidization, allopatric diploid divergences, recent interspecific hybridization and allopolyploidization precipitated by anthropogenic translocation and cultivation (Hughes et al. 2007; Govindarajulu et al. 2011a, 2011b). This complex myriad of evolutionary mechanisms influencing the history of *Leucaena* presents challenges for reconstructing an accurate phylogeny. In addition to reviewing past work on *Leucaena*, we summarize on-going and recently published genomic work and the utility of these new genomic data for basic and applied research on *Leucaena*.

Taxonomy and morphology

The genus *Leucaena* was first described in 1842 when Bentham (1842) transferred *Acacia glauca*, *A. pulverulenta*, *A. diversifolia* and *A. trichodes* to this newly recognized genus. Subsequent work by Bentham (1846; 1875), Standley (1922), Britton and Rose (1928), Brewbaker (1987a), Harris et al. (1994), Zárate (1994), Hughes (1998a) and Govindarajulu et al. (2011a) has all contributed to the modern circumscription of 24 species within the *Leucaena* genus.

The genus is placed in the informal Leucaena group alongside *Desmanthus*, *Kanaloa* and *Schleinitzia* within the mimosoid clade of the newly re-circumscribed legume subfamily Caesalpinioideae (LPWG 2017). Anther and pollen morphology as well as chloroplast and nrDNA ITS sequence data have been used to determine generic relationships within the Leucaena group (Hughes 1997; 1998b), which is part of a larger clade including the informal *Dichrostachys* group, plus the genera *Prosopidastrum*, *Piptadeniopsis* and *Mimozyganthus* (Hughes et al. 2003; Luckow et al. 2005; LPWG 2017).

All 24 species of *Leucaena* are woody, single or multi-stemmed trees or shrubs ranging from 4 to 25 m tall. The shoots are always free of spines or prickles. Terminal shoots can either be terete or ridged with corky fiber bundles. Leaves in *Leucaena* are always stipulate, alternate and bipinnate, but show significant and conspicuous quantitative variation within and between species in terms of numbers of pairs of pinnae per leaf and leaflets per pinna and leaflet size. Many species exhibit nyctinasty (circadian-based 'sleep' movement) in their leaflets and pinnae; however, seismonasty (touch sensitivity) does not occur in *Leucaena* (Hughes 1998a).

Extrafloral nectaries are found on various parts of the leaves of all *Leucaena* species. These nectar-secreting glands mediate mutualisms with ants for protection against herbivory, and are common across the majority of mimosoid legume genera (Marazzi et al. 2013). The morphology and arrangement of these structures help distinguish some *Leucaena* species from others (Hughes 1998a).

The stamen filaments are generally yellow, white or pink, and flowers are borne in globose or subglobose capitula (head-like clusters) that are variously arranged on flowering shoots. Pods generally arise in clusters of 1–15, but sometimes as many as 45 from a single capitulum. *Leucaena* seeds typically have circular to ovate or ellipsoid shape and are dorsi-ventrally flattened (Hughes 1998a).

Cladistic analyses of morphological data (Hughes 1998a) revealed limited support for a number of groups. For example, the *Leucaena esculenta* group shares thick and corky bark with gray-metallic surfacing, while the closely related *L. retusa* and *L. greggii* share stipitate extrafloral nectaries. Quantitative analyses of leaf traits (number of pairs of pinnae, number of pairs of leaflets and size of leaflets) show clear patterns of morphological intermediacy in hybrids, including a dosage effect due to ploidy (Sorensson 1993; Hughes and Harris 1994, 1998; Hughes 1998b), suggesting tight genetic control of quantitative leaf morphology.

Crossability among species

Through a massive series of artificial intra- and interspecific crossing experiments, Sorensson and Brewbaker (1994) investigated the potential to generate hybrids as well as the mechanisms and degree of incompatibility within and among species. At that time, just 16 species (15 published and 1 unpublished) were recognized in the genus. Of the 120 possible 2-way mating combinations, 118 were artificially hybridized and 31 of the 32 possible self- and interspecific mating combinations tested (Sorensson and Brewbaker 1994). An impressive 58,218 floret emasculations and hand-pollinations were made, with 77% of 118 two-way combinations and 61% of 232 one-way combinations producing viable seed, demonstrating high crossability among species and the tremendous scope for the use of crossing in breeding work to generate novel hybrids, which have dominated *Leucaena* improvement programs to date (Brewbaker et al. 1989; Brewbaker and Sorensson 1990). Furthermore, the predominant factor in interspecific incompatibility was variation in ploidy between parents, whereas gametophytic self-incompatibility was noted at the intraspecific diploid level. Crossability among morphologically, genetically, geographically and even chromosomally distinct diploid species is consistent with a predominant mode of allopatric, rather than sympatric, speciation (Govindarajulu et al. 2011a).

Variation in chromosome number and genome size: Paleopolyploidy, diploidization and neopolyploidy

Most diploid mimosoids have a chromosome complement of 2n = 26 (e.g. Santos et al. 2012), suggesting a base number of x = 13 for mimosoids. However, the 'diploid' species of *Leucaena*, whose chromosome numbers have been counted, have 2n = 52 or 56 (Pan and Brewbaker 1988; Palomino et al. 1995; Cardoso et al. 2000; Schifino-Wittmann et al. 2000), which is consistent with *Leucaena* having experienced an ancient polyploidization (paleopolyploidization), i.e. whole genome duplication, prior to the diversification of the modern 'diploid' lineages. Nevertheless, these species are typically referred to as 'diploids' because they show primarily disomic, rather than tetrasomic, patterns of inheritance (Pan 1985; Sorensson and Brewbaker 1989).

Furthermore, genome size data (Palomino et al. 1995; Hartman et al. 2000; Govindarajulu et al. 2011b) for 24 species of *Leucaena* suggest that *L. macrophylla* has the smallest genome of all legumes (data.kew.org/cvalues) and that other diploid *Leucaena* species also have relatively small genomes ranging from 0.31 to 1.65 pg/1C. Although some of the absolute sizes of these genomes are inconsistent with subsequent unpublished estimates for all 19 diploid taxa (Trujillo and Bailey, unpublished data), both sources are consistent with typical 'diploid' genomes rather than full tetraploid complements that might have been retained from the paleopolyploidization event. Ultimately the combined evidence from chromosome numbers, disomic inheritance and genome sizes suggests extensive genomic diploidization following an ancestral paleopolyploidization along the stem lineage of *Leucaena* (Govindarajulu et al. 2011b; Figure 1).

By contrast, the tetraploid *Leucaena* species have 2n = 104 or 112 chromosomes with genome sizes (Palomino et al. 1995; Hartman et al. 2000; Govindarajulu et al. 2011b) close to the sum of their parental complements (see below), consistent with little diploidization in the modern 'tetraploid' lineages, suggesting that either the mechanism of diploidization is not functioning to any great degree in these tetraploid lines, or these tetraploid taxa arose recently, offering insufficient time for diploidization to have significantly reduced genome sizes (Govindarajulu et al. 2011b).

Phylogenetics of *Leucaena*

Early assessments of relationships among species of *Leucaena* involved analysis of morphological, cytological and crossability evidence (Zárate 1984, 1994; Brewbaker 1987b; Pan and Brewbaker 1988; Hughes 1998b). The first molecular phylogenetic investigation of *Leucaena* (Harris et al. 1994) used cpDNA RFLP data for 22 species and showed for the first time 3 main clades of diploids. However, conflict between the cpDNA gene tree and morphology and cytology suggested that cpDNA might have been influenced by plastome capture, raising doubts about this initial cpDNA gene tree as a species tree.

A clearer understanding of species limits plus the addition of nrDNA ITS sequence data and a rescoring of the cpDNA restriction fragment length polymorphism (RFLP) data (Hughes et al. 2002) presented relationships that were in agreement with the previous cpDNA RFLP study (Harris et al. 1994) in resolving 3 main clades of diploid species. However, within these 3 clades, bootstrap support values (particularly in Clade 1) remained low. To address this problem, an approach based on random amplification of polymorphic DNA was used to develop a set of anonymous low-copy nuclear loci and these were sequenced (Bailey et al. 2004) to further estimate relationships among species (Hughes et al. 2007; Govindarajulu et al. 2011a, 2011b). Govindarajulu et al. (2011a), using 59 accessions representing all diploid taxa, recovered the 3 clades established from earlier molecular work as well as a more robust estimate of interspecific relationships. Results from this analysis also provided strong evidence for allopatric divergence as the predominant mode of speciation among the diploid species (as noted above).

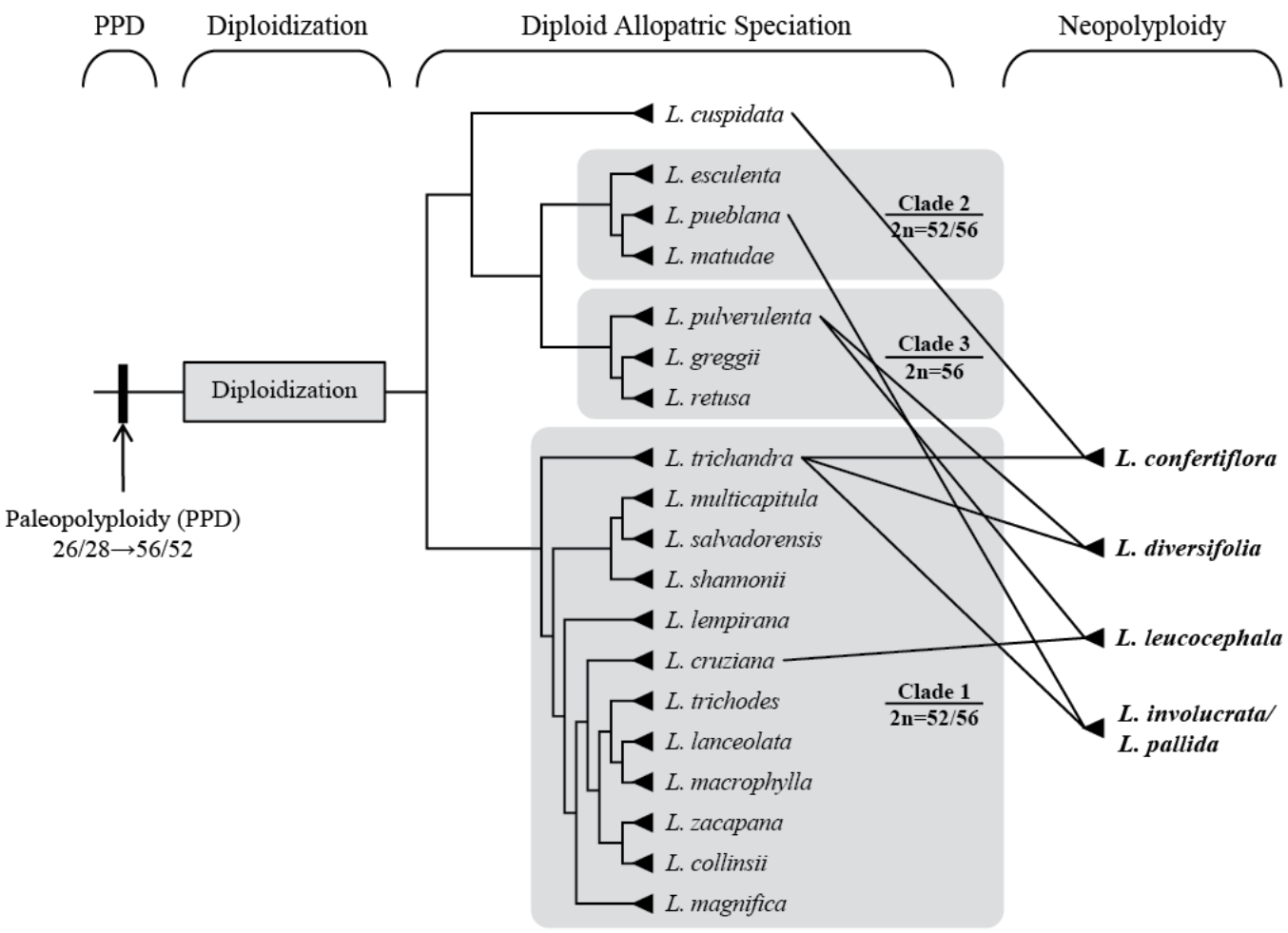

Figure 1. The evolutionary dynamics of polyploidy in *Leucaena*. The diploid phylogeny summarizes relationships recovered by Govindarajulu et al. (2011a). Multiple accessions of each species are collapsed to single terminals. Figure reprinted from Govindarajulu et al. (2011b) with permission from Wiley Company.

In these studies, multiple diploid populations were sampled using AFLPs to explore species boundaries on a scale not possible with morphological or cytological characters alone (Govindarajulu et al. 2011a). The resulting population genetic results supported the previously recognized taxonomy (Hughes 1998a), except for *L. lanceolata*, which was shown to be polyphyletic leading to the addition of *L. cruziana* as a species distinct from *L. lanceolata*, and upranking of *L. collinsii* subsp. *zacapana* as a distinct species (*L. zacapana*) (Govindarajulu et al. 2011a).

With these clarifications of species limits and the accumulated phylogenetic evidence, there is strong support for recognizing 3 major clades of diploids: Clade 1 (*L. collinsii*, *L. cruziana*, *L. lanceolata*, *L. lempirana*, *L. macrophylla*, *L. magnifica*, *L. multicapitula*, *L. salvadorensis*, *L. shannonii*, *L. trichandra*, *L. trichodes* and *L. zacapana*); Clade 2 (*L. esculenta*, *L. matudae* and *L. pueblana*); and Clade 3 (*L. greggii*, *L. retusa* and *L. pulverulenta*), with little evidence of homoploid hybridization among or within these clades (Govindarajulu et al. 2011a). The position of the other diploid species *L. cuspidata* and the relationships among closely related species in Clade 1 remain poorly resolved, but forthcoming phylogenetic analyses using much larger DNA sequence data sets (plastomes and nuclear genes from transcriptomes) across species will likely resolve these last remaining phylogenetic questions. With a few minor exceptions, the 3 diploid clades occupy largely allopatric distributions: Clade 1, the most widespread, is distributed from northern South America through Central America, south-central Mexico and along the Pacific coast of Mexico as far north as Sonora in lowland seasonally dry tropical forests; Clade 2 is found in inland regions of the south-central Mexican highlands and seasonally dry valleys mainly south of the Mexican volcanic

axis; and Clade 3 has the most northerly distribution in northeast Mexico (north of the central volcanic axis) extending into southern Texas and adjacent New Mexico in the USA.

Serendipitous hybridization and polyploidy

Allopatric distributions of diploid sister species are consistent with geographical isolation and predominantly allopatric diploid speciation (Govindarajulu et al. 2011a). However, all 5 tetraploid species of *Leucaena* show clear evidence of hybrid (i.e. allopolyploid) origins, implying sympatry of their putative diploid parental species, but sympatry appears to be rare among wild diploid populations. Indeed, evidence suggests that each allotetraploid resulted from crosses between species placed in different diploid clades, which themselves have distinct geographies, further emphasizing the lack of sympatry among diploid species in the wild (Govindarajulu et al. 2011b).

Figure 2 illustrates the hypothesized origins of each allotetraploid species (Govindarajulu et al. 2011b). Here we refer to parental lines in terms of extant species; however, if the crosses were much older, the parental line would have been akin to, but not necessarily the same as, the modern species. Three of the 5 allotetraploids include *L. trichandra* as the putative paternal diploid parent crossed with *L. pulverulenta* to form tetraploid *L. diversifolia* and a species of the *L. esculenta* group to form *L. involucrata* and *L. pallida*. While *L. pallida* and *L. involucrata* may have the same polyploid origin, minor differences in morphology as well as allopatric distributions suggest these are 2 distinct species. The fourth *L. trichandra*-derived tetraploid, *L. confertiflora*, has *L. trichandra* as the putative maternal parent and *L. cuspidata* as the paternal line. Repeated involvement of *L. trichandra* in the origins of 4 of the 5 tetraploids, particularly on the paternal side, is consistent with its high propensity to produce unreduced pollen grains, its wide geographical distribution and early signs of its use as a human food source (Govindarajulu et al. 2011b). The fifth tetraploid species, the widely translocated and pantropically cultivated and naturalized *L. leucocephala* is derived maternally and paternally from *L. pulverulenta* and *L. cruziana*, respectively.

The divergent hybrid origin of each tetraploid lineage has raised interesting questions about the likely origin(s) of these taxa. The available evidence suggests that at least some of these tetraploid species may be the product of serendipitous backyard hybridization via juxtaposition in informal cultivation in central Mexico over the last 6,000 years (Hughes et al. 2007; Govindarajulu et al. 2011b). Evidence consistent with this anthropogenic backyard allopolyploid formation hypothesis includes the aforementioned predominance of allopatry among wild diploids, limited genomic diploidization of tetraploids suggesting recency of the tetraploids (see above), and archeological evidence that suggests the oldest seeds of tetraploid *Leucaena* date from about 1,500 years ago, long after the first appearance of *Leucaena* seed remains, this despite the predominant cultivation and use of allotetraploids in backyard gardens today (Hughes et al. 2007; Govindarajulu et al. 2011b).

Several other putative spontaneous polyploid hybrids have been discovered and documented across south-central Mexico. These are also thought to have arisen following juxtaposition of their parents in cultivation. Two of these have been named as hybrid species. First, the named hybrid taxon, *L. ×mixtec*, a putative triploid between tetraploid *L. leucocephala* and diploid *L. esculenta*, is a relatively common tree across south-central Mexico (Hughes and Harris 1998). As expected for a triploid, these *L. ×mixtec* hybrids are sterile and each individual thus likely represents a de novo F1 spontaneous hybrid. A second named hybrid taxon, *L. ×spontanea*, a putative hybrid between tetraploid *L. leucocepahala* and *L. diversifolia*, occurs as scattered individuals wherever these 2 species occur together (Hughes and Harris 1998). Finally, a few individuals of a putative hybrid between *L. leucocephala* and *L. confertiflora* have

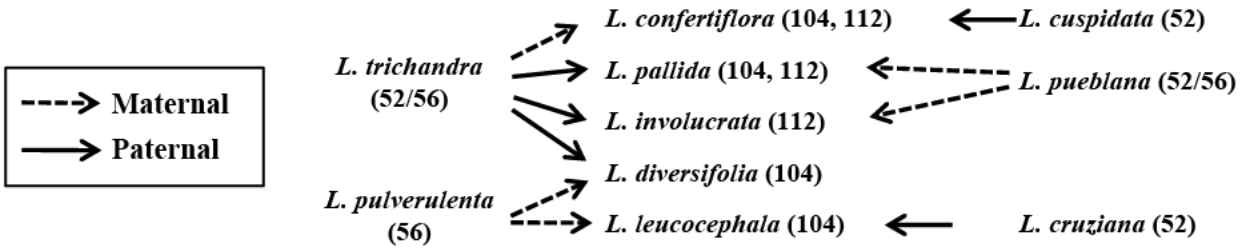

Figure 2. Inferred pattern of tetraploid origins from diploid progenitors. Maternal origins are based on findings from cpDNA and congruent resolution in nuclear-derived gene trees. Paternal origins derive from the divergent placement of nuclear-derived sequences in comparison with the inferred maternal origins. Figures reprinted from Govindarajulu et al. (2011b) with permission from Wiley Company.

been documented, also in south-central Mexico (Hughes et al. 2007). The full extent of spontaneous interspecific hybridization in south-central Mexico remains to be fully investigated.

As a wider range of *Leucaena* species are cultivated on an ever wider scale, continued spontaneous hybridization and generation of new hybrids are likely, adding further complexity to an already complex picture of polyploidy and interspecific hybridization.

Developing genomic resources

Future applied and basic research on *Leucaena* will benefit greatly from recent and ongoing research to sequence a *Leucaena* genome and generate transcriptome data for all species. Diploid *L. trichandra* was selected as the species for genome sequencing because it is the putative progenitor of 4 of the 5 tetraploid species. Table 1 summarizes available *Leucaena*-associated NCBI Sequence Read Archive (SRA) resources, including genomic DNA reads representing organellar and nuclear genomes, as well as a variety of transcriptomic data (RNA-seq) from multiple species. Below we briefly review some of the pertinent findings from these studies and outline ongoing work.

The chloroplast genomes for *Leucaena trichandra* and other mimosoids were sequenced by Schwarz et al. (2015) and Dugas et al. (2015), resulting in resources relevant to the understanding of variation in coding and non-coding chloroplast sequence, cpDNA genome structure and RNA editing. Similarly, Kovar et al. (2018) recently published a mitochondrial genome (*L. trichandra*) with discussion on the origin of mtDNA DNA variation and mitochondrial RNA-editing across the genus. In these studies, the mimosoid organellar genome(s) is considerably larger than their papilionoid legume counterparts. The authors discuss some of the sources and mechanisms behind this

Table 1. NCBI Sequence Read Archive materials available for public use.

SRA Accession	Species	Library Source	Data type
SRX2719625	*L. trichandra*	Genomic DNA	PacBio raw reads from the mitochondrial genome assembly - gDNA
SRX2719624	*L. trichandra*	Genomic DNA	4kb insert mate pair library - set 1
SRX2719623	*L. trichandra*	Genomic DNA	4kb insert mate pair library - set 2
SRX1341614	*L. trichandra*	Genomic DNA	300bp Illumina PE library data
ERX386816	*L. leucocephala*	Genomic DNA	PE Illumina data for microsatellite development
ERX386817	*L. leucocephala*	Genomic DNA	PE Illumina data for microsatellite development
SRS2110148	*L. pueblana*	Genomic DNA	100bp Illumina PE library data
SRX625626	*L. leucocephala*	Genomic DNA methyl CpG depleted	Illumina PE library
SRX625625	*L. leucocephala*	Genomic DNA methyl CpG depleted	Illumina PE library
SRX625623	*L. leucocephala*	Methyl CpG study untreated control	Illumina PE library
SRX886540	*L. leucocephala*	Metagenomic soil sample	
SRX2719621-SRX2719622	*L. cuspidata*	Transcriptomic	RNA-seq library data - rRNA depleted libraries 1 & 2
SRX2719619- SRX2719620	*L. cruziana*	Transcriptomic	RNA-seq library data - rRNA depleted libraries 1 & 2
SRX2719617-SRX2719618	*L. pulverulenta*	Transcriptomic	RNA-seq library data - rRNA depleted libraries 1 & 2
SRX2719615-SRX2719616	*L. trichandra*	Transcriptomic	RNA-seq library data - rRNA depleted libraries 1 & 2
SRX2719613-SRX2719614	*L. leucocephala*	Transcriptomic	RNA-seq library data - rRNA depleted libraries 1 & 2
SRX2719611-SRX2719612	*L. esculenta*	Transcriptomic	RNA-seq library data - rRNA depleted libraries 1 & 2
SRX1282083-SRX1282084	*L. leucocephala* K636	Transcriptomic	RNA-seq library data - rRNA depleted library
SRR2517688	*L. leucocephala*	Transcriptomic	RNA-seq library of shoots
SRR2517689	*L. leucocephala*	Transcriptomic	RNA-seq library of roots

size variation. The associated RNA-seq and gDNA-seq genomes are essential prerequisites for the development of organellar-derived species-specific markers and for gene expression studies at the organellar level.

In addition to organellar genomic resources, the Bailey Lab at New Mexico State University is currently completing a draft nuclear genome, based on PacBio and Illumina sequence data for the diploid *L. trichandra* (Bailey et al. unpublished data – available, with stipulations on publication priority/conflicts from the authors on request). Current analysis and annotations on the genome suggest that *L. trichandra*, and presumably other 'diploid' *Leucaena*, retain considerable evidence of the paleotetraploidization event that predates the divergence of 'diploid' *Leucaena* (Figure 1).

In addition to these resources, the Bailey Lab (NMSU) and the Borthakur Lab (UNH Manoa) are continuing to work on a number of resources, including an investigation of plant transcript response to psyllid feeding in *L. cruziana* (Lakshman et al. in prep.) and the Bailey Lab has transcriptomic and raw genomic data available from *Leucaena* psyllids. Like some of their relatives (www.ncbi.nlm.nih.gov/genome/genomes/867?genome_assembly_id=31561), these psyllid genomes display considerable bias in GC content that complicates the use of Illumina data to assemble a full genome.

Future directions

These new genomic data are providing new insights into the phylogenetic relationships among diploid species (Abair et al. in preparation) plus the origins of the allotetraploids, as well as tools and resources for germplasm improvement. While the basic phylogenetic framework and likely polyploid parentages are now fairly well established, the details of these polyploid origins in terms of where, when and how many times they happened remain poorly understood. The emerging genomic data resources provide access to unlimited genetic markers that could be used to test for multiple independent origins for each of the 5 *Leucaena* tetraploids, and most notably the globally important *L. leucocephala* and its morphologically variable taxonomic subspecies. A key element in future work is likely to involve much denser sampling of accessions of tetraploids and their diploid parents to fully reveal the complexities of this extensive hybrid and polyploidy series.

These new genomic tools and resources, alongside a better understanding of the evolutionary history of *Leucaena*, also present exciting new opportunities for *Leucaena* genetic improvement and breeding programs, including efforts to develop seed or sterile lines with low potential for invasiveness, decreased mimosine concentration and traits that improve their utility in difficult environments (salinity, cold, drought, etc.).

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(Note of the editors: All hyperlinks were verified 24 April 2019.)

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ILC2018 Keynote paper*

Sterile leucaena becomes a reality?

¿Leucaena estéril será una realidad?

HAYLEY E. MCMILLAN[1*], GUOQUAN LIU[1*], H. MAX SHELTON[1], SCOTT A. DALZELL[1], IAN D. GODWIN[1], HARSHI GAMAGE[1], CLEO SHARMAN[2] AND CHRISTOPHER J. LAMBRIDES[1]

[*] *Joint senior authors*
[1] *School of Agriculture and Food Sciences, The University of Queensland, Brisbane, QLD, Australia.* agriculture.uq.edu.au
[2] *Mt St Michael's College, Ashgrove, QLD, Australia.* msm.qld.edu.au

Abstract

A research program to develop sterile leucaena has commenced to enhance red-meat production in additional regions of Australia including Western Australia, Northern Territory and New South Wales, where growing seeded leucaena is not currently permitted or encouraged. In this study we report on the development of methodology using a mutagenizing agent, EMS (ethyl methanesulfonate), to cause mutations in the self-fertile commercial leucaena cultivar, Redlands. Several experiments to determine the optimum rate of EMS have been completed and first generation mutagenized plants (M_0) established in the field at Redlands Research Station, Cleveland, Queensland, Australia. An EMS concentration of 0.35% applied to germination paper proved the best method to achieve a target emergence percentage of 50%. To date, 27 of 179 mutagenized M_0 seedless plants are considered to be putatively sterile. A further 1,200 M_0 plants have been established in the field providing an even greater chance of identifying sterile leucaena plants with the desired forage quality and psyllid-resistance attributes.

Keywords: Ethyl methanesulfonate, mutagenesis, seedless, shy seeding.

Resumen

Un programa de investigación para desarrollar variedades de leucaena estériles fue iniciado para beneficiar la producción de carne roja en diferentes regiones de Australia, incluyendo Western Australia, Northern Territory y New South Wales, donde actualmente no se permite o fomenta el cultivo de leucaena por su potencial riesgo como maleza. En este estudio informamos sobre el desarrollo de una metodología utilizando un agente mutagenizante, EMS (metanosulfonato de etilo), para generar mutaciones en un cultivar comercial de leucaena autofértil, cv. Redlands. Se completaron varios experimentos para determinar la tasa óptima de EMS y se establecieron plantas mutagenizadas de primera generación (M_0) en el campo de experimentación Redlands, Cleveland, Queensland, Australia. Se encontró que una concentración de EMS del 0.35% aplicado al papel de germinación es el mejor método para lograr un porcentaje de emergencia del 50%. Hasta la fecha, 27 de 179 plantas M_0 mutagenizadas sin semillas se consideran supuestamente estériles. Se han establecido otras 1,200 plantas M_0 en el campo, lo que brinda una posibilidad aún mayor de identificar plantas de leucaena estériles al tiempo que se retienen los atributos deseados como alta calidad forrajera y resistencia a los psílidos (insectos de la familia Psyllidae).

Palabras clave: Metanosulfonato de etilo, mutagénesis, plantas sin semillas, semillación escasa.

Introduction

In Northern Australia, leucaena (*Leucaena leucocephala* ssp. *glabrata*) is planted in single or double hedgerows several meters apart with a perennial C4 grass planted in the inter-row, and occasionally with a C3 grass in the winter months. This legume-grass pasture system is highly productive for grazing cattle in the >600 mm rainfall zone, with producers reporting greater liveweight gains and profitability compared with other tropical pastures (Shelton

Correspondence: Christopher J. Lambrides, School of Agriculture and Food Sciences, The University of Queensland, Brisbane, QLD 4072, Australia. Email: chris.lambrides@uq.edu.au

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and Dalzell 2007; Radrizzani et al. 2010). However, in some regions of Australia leucaena is considered a serious weed, primarily because of the seediness of commercial cultivars, but also because of the wide historic distribution of the non-commercial weedy type *L. leucocephala* ssp. *leucocephala* (Shelton et al. 2003; Walton 2003). This weedy non-commercial subspecies has been present in Australia since the late 1800s and pre-dated agricultural production with subspecies *glabrata* (White 1937). In some regions of Australia, e.g. pastoral lease-hold land in Western Australia and Northern Territory, establishment of commercial leucaena plantations is forbidden due to the perceived environmental weed risk. Consequently, there is a need to develop seedless (sterile) cultivars in order for these regions to share the benefits of leucaena-grass pasture systems.

There are several non-transgenic approaches to developing sterility in leucaena including: cytoplasmic male sterility (CMS) (Saxena and Kumar 2003); interspecific hybridization (e.g. sterile triploids) (Sorensson and Brewbaker 1994); and induced sterility via mutagenesis (Blomstedt et al. 2012). The key to the success of any of these methods will be the ability to produce enough sterile propagules to be viable for commercial use.

Cytoplasmic male sterility

CMS is a genetic system that enables plant breeders to breed hybrid varieties in a range of species, including sunflower, maize, sorghum, rice and pigeon pea to name a few. An advantage of this system is that potentially sterile varieties can be released to producers in the form of seed. To date, no CMS systems have been identified in leucaena, although there have been recent reports of CMS being discovered in another forage legume, pigeon pea (*Cajanus cajan*) (Saxena and Kumar 2003). This finding in pigeon pea is significant because previously no CMS system had been identified in any legume species. CMS may be developed in leucaena by intercrossing different *Leucaena* spp. and possibly through mutagenesis.

Interspecific hybridization - Sterile triploids

The genus *Leucaena* contains diploid and tetraploid species that can potentially be crossed to develop sterile triploid leucaena plants (Sorensson and Brewbaker 1994). Diploid leucaena species, e.g. *L. collinsii*, *L. greggii*, *L. retusa*, *L. magnifica* and *L. macrophylla*, are predominantly self-incompatible (SI) meaning that individual trees are self-sterile and require pollen from another tree to set seed. Conversely, many of the tetraploid *Leucaena* species, e.g. *L. leucocephala*, *L. diversifolia* and *L. confertiflora*, are self-compatible (SC) so that individual trees are self-fertile and do not need to be outcrossed to another individual to set seed. All of the commercial leucaena cultivars grown in Australia today are from the tetraploid species *L. leucocephala*, and as such are SC. Knowledge of the mating systems can help facilitate the planting of crossing blocks that can be used to develop sterile triploids. This system would require a single genotype of a diploid plant to be cloned and planted in a production field as a female alongside a tetraploid pollinator, e.g. a current commercial cultivar, to be used as the male pollen donor. Seed harvested from the diploid female parent would be sold to producers for commercial use. Alternatively, if sterile triploid seeds can be made by hand crosses, the resultant plants can be multiplied by vegetative propagation and distributed to producers for commercial use. One caveat here is that pollen mentoring has been demonstrated in leucaena seed orchards (University of Queensland tried this approach to make KX2 F1 hybrid seed), which resulted in pure female seed contamination.

Interspecific hybridization - Other

One tetraploid leucaena species that is SI and not SC is *L. pallida* (Sorensson and Brewbaker 1994) and this species was used as a source of psyllid resistance in several previous research projects with the breeding objective of delivering psyllid-resistant cultivars. During one of such breeding programs to develop psyllid-resistant leucaena, progeny from the interspecific cross *Leucaena pallida* × *L. leucocephala* were developed and selected for high IVDMD (in vitro dry matter digestibility), psyllid resistance and self-compatibility. Eventually, breeding line 12 met all the criteria and was released as cv. Redlands. However, of the 40–50 breeding lines that were under consideration at the time, several produced very little seed and were considered shy-seeding. Some earlier generations of these lines were observed to produce no seed. Consequently, these lines are potentially useful for the purpose of breeding sterile leucaena. For these 'sterile' plants to be useful on a commercial basis they would need to be propagated clonally. It should be noted that these lines were never conclusively proven to be sterile. They would need to be evaluated in a wide range of environments to make sure photoperiod × temperature interactions did not trigger flowering and seed set outside of the Brisbane environment where the breeding project took place.

Mutagenesis

Inducing sterility by mutagenesis is a relatively easy and direct way of producing sterility in plants by exposing seeds to a mutagen. Ethyl methanesulfonate (EMS) is a

chemical mutagen that is often the agent of choice because it has caused high rates of sterility in target plant species (Kurowska et al. 2011). A characteristic of EMS as a mutagen is that it causes single base pair mutations, e.g. GC to AT or AT to GC transitions. While many genetic changes may occur using this mutagen, not all result in a detectable phenotype, and consequently, it is always necessary to check that other characteristics, e.g. quality attributes, have not been altered in candidate plants. In the study reported here we will describe a new methodology for using EMS to create sterile leucaena plants. We have now completed several experiments with EMS applied to seed of the commercial cultivar, Redlands. The objective of these initial experiments was to determine what concentration of EMS would cause an emergence percentage of about 50%, to find a general relationship between EMS treatment and mutation rate and to identify putative sterility in any mutagenized plants. When using mutagens it is generally accepted that some seed death will occur and a 50% emergence rate is generally considered a good compromise between seed death and an adequate mutation rate (Blomstedt et al. 2012).

Materials and Methods

Mutagenesis

Experiment 1. Seeds of cv. Redlands (bred from the interspecific hybrid *Leucaena pallida* × *L. leucocephala*) were surface-sterilized using a 30-s rinse with 10% bleach solution before being treated with a chemical mutagen, EMS (ethyl methanesulfonate). Six concentrations of EMS (0.00, 0.10, 0.25, 0.50, 0.75 and 1.00% w/v) and 2 periods of imbibition (16 and 40 hours) were used to treat 300-seed batches of pre-scarified seed. Seeds were scarified with a sharp blade by removing a small piece of the testa at the end opposite from the radicle. For each treatment, seeds were placed in glass beakers set on a gentle rocking platform and completely immersed in 500 ml solutions of the EMS treatment.

Experiment 2. Since the emergence rate of all treated seed in Experiment 1, including the control, was much lower than expected, a second experiment (Experiment 2) was conducted. Seeds were treated on germination paper that had been placed in sterilized germination trays and dampened with the same EMS concentrations used in Experiment 1. Germination trays were kept in the dark for 3 days before the seed was rinsed with deionized water.

Experiment 3. For this experiment, conditions were the same as for Experiment 2 except that the following EMS concentrations were tested to identify the concentration that resulted in a 50% emergence rate: 0.00, 0.25, 0.30, 0.35, 0.40, 0.45 and 0.50% w/v.

Field planting

All germinated seeds (M_0 generation) were then sown in small forest tubes filled with a peat mix when the radicle was 2–10 mm long. (NB. M_0 is the first after the mutagen is applied, and seed from M_0 plants will be M_1 etc.). The seedlings were placed in a shade house until field planting when they were 20–30 cm tall. Plants of Experiment 1 (108) were sown in the field on 18 December 2017 at Redlands Research Station, Cleveland, Queensland, Australia in rows 1.0 m apart, and with a 0.5 m intra-row spacing. Control plants (0.00% EMS) of cv. Redlands were planted approximately every 30 plants along the row. In addition, 546 M_0 plants from Experiment 2 and 605 M_0 plants from Experiment 3 were planted at Redlands Research Station on 1 February and 10 April 2018, respectively, using the same procedures described for Experiment 1.

Phenotyping

One hundred and eighty-three surviving plants of Experiment 1 (including controls plants) were phenotyped on 3 occasions (270, 299 and 370 days after field planting) for mutations using a simple phenotypic scoring system. An estimate of mutation rate was calculated by scoring each plant relative to control plants for several attributes, including: flower set (1 = no flowers, 2 = 1–10 flowers, 3 = 11–50 flowers and 4 = >50 flowers); pod set (1 = no pods, 2 = 1–10 pods, 3 = 11–50 pods and 4 = >50 pods); flower color (1 = white, 2 = not white); plant habit (1 = arboreal, 2 = non-arboreal and 3 = prostrate); vigor (1 = normal and 2 = reduced vigor); and seedling viability (1 = viable and 2 = dead). The mutation rate was considered an underestimate since some single-base mutations at the DNA level would not have resulted in an observably different phenotype and the number of seeds that failed to germinate during the treatment period were not considered for the calculation.

Results

Effects of EMS concentration and period of imbibition on emergence

In Experiment 1, the emergence rate did not exceed 16% for any treatment because the durations of seed imbibition were too long (16 and 40 h). Most seeds rotted in the aqueous solutions of the EMS and the effects increased with the period of imbibition (Figure 1). Therefore, in subsequent

experiments, seeds were placed on germination paper moistened with solutions of EMS, making sure that anaerobic conditions were avoided. As a result of the modified protocol, emergence rates in Experiment 2 were as high as 80%. Based on the results of Experiment 2, the target of 50% emergence rate of treated seed was hypothesized to fall between the 0.25 and 0.50% EMS treatments. This was tested in Experiment 3 by including additional rates of EMS within the range of 0.25– 0.50%. The combined results of Experiments 2 and 3 are shown in Figure 2 where a curvilinear relationship between EMS concentration and average emergence was observed; 50% emergence occurred at an EMS concentration of 0.35%.

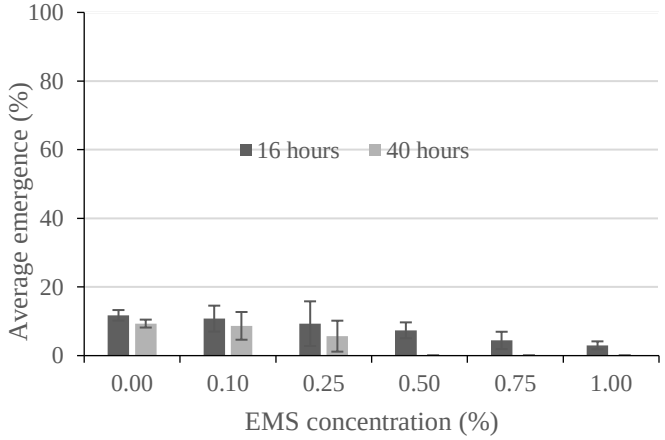

Figure 1. Experiment 1. The effect of increasing ethyl methanesulfonate (EMS) concentration and duration of seed imbibition on average emergence of cv. Redlands. Seeds treated in solutions of EMS. Data shown are means of all reps (n=36).

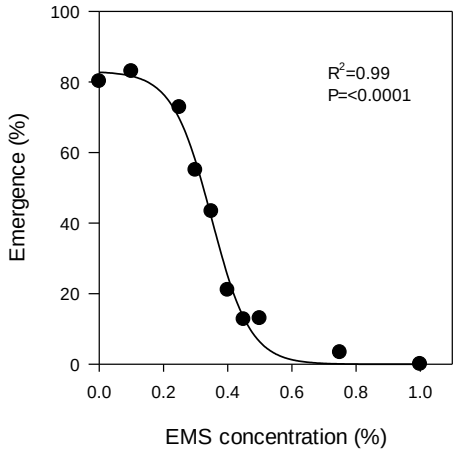

Figure 2. Experiments 2 and 3. The effect of increasing EMS concentration on emergence of cv. Redlands. Seeds were placed on germination paper saturated with solutions of the EMS treatment. Data shown are means of all reps (n=39).

Effects of EMS treatment on mutation rate

On average, the control plants of cv. Redlands were assigned the following scores: flower set = 4 (>50 flowers); pod set = 4 (>50 pods); flower color = 1 (white); plant habit = 2 (non-arboreal); vigor = 1 (normal); and seedling viability = 1 (viable). Figure 3 shows the relationship between EMS concentration and mutation rate and, not surprisingly, the highest rate of mutation based on assessments of phenotype were induced by 1.00% EMS, the highest rate of EMS used in these experiments. However, the trade-off was that emergence rate of seeds treated with 1.00% EMS was <5% in Experiment 1 and 0% in Experiment 2.

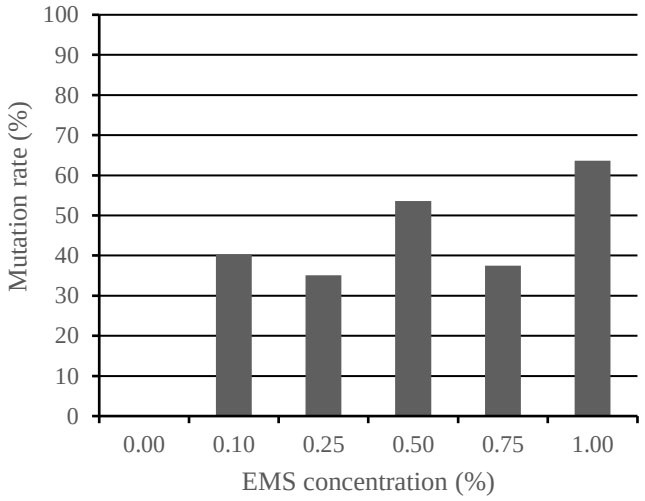

Figure 3. Experiment 1. The effect of increasing ethyl methanesulfonate (EMS) concentration on mutation rate, as measured by observable phenotype. Plants were assessed in a field planting at Redlands Research Station, Cleveland in Autumn-Spring 2018. Data collected from 183 plants including controls (3 plants died).

Effects of EMS treatment on flower and pod set

Based on field assessments made on 3 separate occasions over a 100 day period, each rate of EMS reduced flower and pod set relative to the control treatment (Table 1). An EMS concentration of 1.00% had the greatest effect with reductions in flower set and pod set of 23–37% and 37–50%, respectively (Table 1). Twenty-seven putatively sterile plants that had a flower set of 4 (>50 flowers) but failed to produce pods were identified (Table 2). Other plants that flowered profusely but produced only a few pods were also identified. Some plants did not flower during the assessment period.

Table 1. The effect of EMS (ethyl methanesulfonate) concentration on average (a) flower set and (b) pod set on Experiment 1 plants. Plants were assessed over a 100 day period (271–370 days after field planting, DAP). Traits were scored using a 1–4 scale as follows: flower set (1 = no flowers, 2 = 1–10 flowers, 3 = 11–50 flowers and 4 = >50 flowers); and pod set (1 = no pods, 2 = 1–10 pods, 3 = 11–50 pods and 4 = >50 pods). Percentage of flower or pod set, relative to the control plants, is given in parentheses. Planting date was 18-19 December 2017.

EMS concentration (%)	No. of plants	(a) Average flower set			(b) Average pod set		
		271 DAP	299 DAP	370 DAP	271 DAP	299 DAP	370 DAP
0.00 (control)	7	4.00 (100)	3.86 (100)	4.00 (100)	2.29 (100)	2.71 (100)	2.14 (100)
0.10	67	3.28 (82)	3.13 (81)	3.93 (98)	1.90 (83)	2.06 (76)	1.82 (85)
0.25	57	3.02 (76)	2.89 (75)	3.53 (88)	1.69 (74)	1.80 (66)	1.62 (76)
0.50	28	3.26 (82)	3.07 (80)	3.48 (87)	1.67 (73)	1.74 (64)	1.59 (74)
0.75	16	2.88 (72)	2.81 (73)	3.38 (85)	1.88 (82)	1.88 (69)	1.63 (76)
1.00	11	2.55 (64)	2.45 (63)	3.09 (77)	1.45 (63)	1.36 (50)	1.27 (59)

Table 2. Pod and seed set scores of 27 putative sterile plants from an EMS (ethyl methanesulfonate) mutagenesis experiment (Experiment 1). The sterile plants were identified from field assessments over a 100 day period (271–370 days after field planting). Traits were scored using a 1–4 scale as follows: flower set (1 = no flowers, 2 = 1–10 flowers, 3 = 11–50 flowers and 4 = >50 flowers); and pod set (1 = no pods, 2 = 1–10 pods, 3 = 11–50 pods and 4 = >50 pods). Planting date was 18-19 December 2017.

EMS Treatment (%)	No. of plants	Flower set	Pod set
0.00	7	4	4
0.01	8	4	1
0.25	7	4	1
0.50	7	4	1
0.75	2	4	1
1.00	3	4	1

Discussion

A method for mutagenesis of leucaena

A major initiative of this study was to develop sterile leucaena using a mutagenesis approach. We have now made significant progress in developing the methodology required to mutagenize seeds of leucaena. An EMS rate of 0.35% was effective at producing the target germination rate of 50% in a single genotype of leucaena (cv. Redlands). Given cv. Redlands was bred from the interspecific hybrid *L. leucocephala* × *L. pallida*, it cannot be assumed that an EMS rate of 0.35% will be suitable for other cultivars/species of leucaena. Consequently, additional genotypes will need to be tested.

Identification of putative sterile plants

Importantly, after field testing 179 mutagenized plants from Experiment 1, we have identified several putative sterile leucaena plants. Plants that flowered but did not set pods are potentially female sterile. Plants that flowered and set a few pods are potentially male sterile or self-incompatible, with flowers on these plants presumably receiving pollen from neighboring plants, resulting in fertilization and pod set. At this point we are unable to be more definitive about the nature of sterility observed in each of these plants. Male sterility will be investigated by testing the viability of freshly collected pollen grains using standard staining and pollen germination techniques.

Although 179 mutagenized plants were assessed in the current study, Experiments 2 and 3 collectively resulted in over 1,200 mutagenized plants. These will be assessed for further sterile candidates in future seasons when all control plants are flowering profusely. Given the high frequency of putative sterile plants from Experiment 1, we anticipate identifying a large number of target candidates in Experiments 2 and 3.

Once the mode of sterility can be determined it will also be necessary to check if desirable attributes (e.g. high IVDMD, psyllid resistance etc.) of the control genotype (cv. Redlands) have been retained. We were also able to identify plants that failed to flower during the 100-d observation period. It will take several seasons, as well as planting at higher and lower latitudes, to determine if these plants will remain flowerless permanently.

Propagation of sterile plants

One challenge of developing sterile plants is being able to economically multiply any candidate for commercial use. Two approaches are being considered to propagate candidate sterile plants. We have started to investigate methods of vegetative propagation, by dipping the ends of freshly cut branches into solutions of commercial, off-the-shelf root-forming hormone, IBA/NAA.

A second more challenging approach is to develop a CMS (cytoplasmic male sterile) system in leucaena. This system is typically used to make hybrid sunflower, sorghum and rice. However, in the case of sterile leucaena, where seed production is to be avoided, R lines that restore fertility of a CMS female A line are not required. Consequently, it would be sufficient to maintain the A line by crossing with a B line counterpart. In this case, the commercial entity grown by leucaena producers from seed would be a sterile A line. It is possible that some of the sterile plants identified in the present study are CMS. The candidate CMS plants will need to be crossed with candidate B line plants (possibly cv. Redlands) and the resultant seed tested for fertility.

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ILC2018 Keynote paper*

Strategies to breed sterile leucaena for Western Australia

Estrategias para el desarrollo de genotipos de leucaena estériles para Western Australia

DANIEL REAL[1], YONG HAN[2], C. DONOVAN BAILEY[3], SAIPRIYAA VASAN[2], CHENGDAO LI[1,2], MARIECLAIRE CASTELLO[1], SUE BROUGHTON[1], ALEXANDER ABAIR[3], SAM CROUCH[4] AND CLINTON REVELL[1]

[1]*Department of Primary Industries and Regional Development, South Perth, WA, Australia.* dpird.wa.gov.au
[2]*School of Veterinary and Life Science, Murdoch University, Murdoch, WA, Australia.* murdoch.edu.au
[3]*Department of Biology, New Mexico State University, Las Cruces, NM, USA.* bio.nmsu.edu
[4]*Department of Primary Industries and Regional Development, Broome, WA, Australia.* dpird.wa.gov.au

Abstract

Strategies to breed sterile leucaena for Western Australia include plant breeding and biotechnology tools to generate sterile lines at both the tetraploid and triploid ploidy levels. For tetraploids, the main target species is the commercial *Leucaena leucocephala*, that is well known for its potential as a high-quality, productive and persistent forage. Gene editing technologies (CRISPR) will be utilized to edit out flowering genes and develop a non-flowering *L. leucocephala* and/or create male/female genic sterile lines of *L. leucocephala*. For triploids, the strategy is to cross tetraploid species (*L. leucocephala* and/or *L. diversifolia*) with diploid species to generate sterile triploid hybrids. The diploid parents will include species that have good forage attributes such as *L. collinsii*, *L. macrophylla*, *L. shannonii* and *L. pulverulenta*. Several of these triploid crosses have already been created by the Department of Primary Industries and Regional Development (Perth, Western Australia) and will be evaluated in the Kimberley and Pilbara regions of Western Australia for their agronomic performance and sterility. Vegetative propagation will be required for the tetraploid gene-edited non-flowering *L. leucocephala*. Triploids can either be vegetatively propagated, once generated, or generated via a seed production nursery.

Keywords: CRISPR, doubled haploids, haploids, plant breeding, tetraploids, triploids.

Resumen

Las estrategias para producir leucaena estéril para el estado de Western Australia incluyen herramientas de fitomejoramiento y biotecnología para generar líneas estériles tanto en el nivel tetraploide como triploide. Para los tetraploides, la principal especie objetivo es la leucaena comercial, *Leucaena leucocephala*, que es bien conocida por su potencial como forraje de alta calidad, productividad y persistencia. Para la producción de líneas estériles, se utilizarán tecnologías de edición genética (CRISPR) para eliminar los genes de floración y desarrollar una *L. leucocephala* que no florece o crear líneas de *L. leucocephala* que tengan esterilidad genética masculina o femenina. Para los triploides, la estrategia es cruzar especies tetraploides (*L. leucocephala* y/o *L. diversifolia*) con especies diploides para generar híbridos triploides estériles. Los padres diploides incluirán especies que tengan buenos atributos forrajeros, como *L. collinsii*, *L. macrophylla*, *L. shannonii* y *L. pulverulenta*. Varios de estos cruces triploides ya han sido creados por el Department of Primary Industries and Regional Development (Perth, Western Australia) y serán evaluados en las regiones de Kimberley y Pilbara en Western Australia respecto a su desempeño agronómico y esterilidad. Para la tetraploide *L. leucocephala* producida por técnicas de CRISPR y que no florece, se requerirán métodos de propagación vegetativa. En cuanto a triploides, una vez generados podrán ser propagados tanto vegetativamente como con base en semilla producida en un vivero de producción de semillas.

Palabras clave: CRISPR, fitomejoramiento, haploides, haploides duplicados, tetraploides, triploides.

Correspondence: D. Real, Department of Primary Industries and Regional Development, South Perth, WA 6151, Australia.
Email: daniel.real@dpird.wa.gov.au

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Introduction

The genus *Leucaena* contains 24 species (Govindarajulu et al. 2011a), but only one species, *L. leucocephala*, has a pantropical distribution. According to Brewbaker (2016), it was collected originally from Mexico by Spanish conquistadors and distributed to other Spanish colonies in the late XVI century and now has a global distribution across tropical and subtropical regions of the world. In the last 60 years, *L. leucocephala* has been the target of modern breeding techniques in USA, Australia and Colombia and several cultivars are commercially available, such as Cunningham (released in 1976), Tarramba (released in 1997), Wondergraze (released in 2010) and Redlands (released in 2017) (Cook et al. 2005; IP Australia 2018).

Leucaena leucocephala is highly valued as a multi-purpose tree for wood and forage production. In the major leucaena-growing area of Australia (central and southeast Queensland), about 130,000 ha of *L. leucocephala* has been planted for forage production in single or twin rows with inter-row spacings of 6–12 m with tropical grasses in the inter-rows (Beutel et al. 2018). Bowen et al. (2018) suggest that a *L. leucocephala*-grass mixture is the most productive forage option for beef production in Queensland.

Unfortunately, the attributes of *L. leucocephala* that make it so successful as a forage, e.g. being long-lived, very productive with high nutritive value, competitive with weeds once established and prolific seeding and seedling recruitment, are the same attributes that are beneficial for a successful environmental weed. Hughes (1998) reported that *L. leucocephala* ssp. *leucocephala*, in particular, is a well-known invasive weed species in more than 20 countries.

While *Leucaena leucocephala* is a permitted species in Western Australia (WA), in the north of the state and, in particular, the Kimberley and Pilbara regions, it has not been approved for use on pastoral land leased from the government due to its potential to become an environmental weed (Munday et al. 2018; Revell et al. 2019). Pastoral lease is the dominant form of land tenure for cattle production in the rangelands of WA, with only very small areas of freehold land.

Breeding a sterile forage that will not set viable seeds within the *Leucaena* genus for use on a pastoral lease would enable the exploitation of the productive potential of leucaena in the region without posing a weed threat. This approach is strongly supported by the Department of Primary Industries and Regional Development of Western Australia and the northern beef industry through Meat & Livestock Australia.

Plant materials

All 24 species of *Leucaena* can potentially be utilized for breeding and possess a wide range of useful characteristics for breeding purposes being derived from widely differing environments, e.g. low to high rainfall, low to high elevation, intense heat to intense cold (even frost), alkaline, neutral and/or acid soils, a range of pests and diseases, low to high nutritive value, etc. (Hughes 1998; Revell et al. 2019). There are 5 tetraploid and 19 diploid species in the genus and there is a high level of inter-specific crossing compatibility among them (Sorensson and Brewbaker 1994).

Three tetraploids and 8 diploid species were selected for their forage potential for the WA breeding program (Table 1).

Table 1. Key descriptive characteristics of the tetraploid and diploid species of leucaena selected for the WA breeding program.

Species	Annual rainfall (mm)	Duration of dry season (months)	Digestibility	Condensed Tannins	Cold tolerance	Psyllid resistance	Acid soil tolerance
Tetraploids							
L. leucocephala	650–3,000	6–7	High	Low	No	No	No
L. diversifolia	1,500–3,500	3–4	Medium	Medium	Yes	Moderate	Yes
L. confertiflora	500–700	7	Low	High	Yes	High	No
Diploids							
L. pulverulenta	700–1,000	5–6	Low	High	No	Moderate	No
L. collinsii	500–700	7	High	Low	No	High	No
L. shannonii	800–1,200	5–6	High	Low	No	Moderate	No
L. macrophylla	700–1,500	4–6	High	Low	No	Moderate	No
L. retusa	500–900	6–7	High	Medium	Yes	High	No
L. greggii	350-500	7	Low	Medium	Yes	High	No
L. trichandra	Variable	Variable	Low to High	Low to High	No	Low to High	Yes
L. trichodes	500–1,000	5–7	High	Low	No	Low	No

Source: Hughes (1998).

Molecular markers and ploidy level

Modern high-throughput genotyping techniques (Mammadov et al. 2012) are now available for plant breeders. We are taking advantage of existing genomic and transcriptomic materials for *Leucaena* (Abair et al. 2019) to develop species-specific high-throughput SNP-based marker systems for all taxa. Such markers will aid in the design of specific interspecific crosses and the validation of seeds subsequently produced.

To identify informative species-specific markers we are employing a 2-step process. First, interspecific variation across the coding regions for 18 of the 19 diploid species is being described via transcriptomic (mRNA-seq) resources. For the 19th species, *L. pueblana*, a lack of available seed resources has resulted in the characterization of overlapping variation using gDNA-seq, rather than RNA-seq data. These RNA and DNA data are being utilized in conjunction with the draft genome from *L. trichandra* (Bailey et al. unpublished data) to conduct variant detection. Accepted uniquely mapped regions are being used to target orthologous loci. The filtered results are then merged for variant calling. Insertions and deletions (Indels) and single nucleotide polymorphisms (SNPs) that pass a minimum mapping quality threshold (phred ≥30) will be retained for post processing and filtered once more for taxonomically informative variants.

Since the RNA-seq resources are derived from just 3 samples per species, the second step towards the development of effective species-specific SNP markers is the screening of available gDNA samples that represent individuals whose identities have been previously confirmed by morphological and molecular approaches (Govindarajulu et al. 2011b). This expanded sampling is critical for the identification of markers that broadly represent each species, rather than subsets of individuals or populations.

Molecular markers will be developed based on the Indel/SNP database and then validated in the *Leucaena* species. The Indel markers resulting in different amplicon sizes can be efficiently analyzed with agarose or polyacrylamide gel electrophoresis. For SNP screening, the KASP (Kompetitive Allele Specific PCR) genotyping assay will be employed, which is a novel competitive allele-specific PCR for SNP scoring based on dual FRET (Fluorescent Resonance Energy Transfer). Taking advantage of low cost, high throughput and high specificity, KASP assay has been used extensively in SNP genotyping studies in the major crops such as wheat (Neelam et al. 2013), rice (McCouch et al. 2010) and barley (Hill et al. 2018). A final set of diagnostic markers with specific polymorphisms amongst the leucaena collection will be filtered and used for identification tests. Early identification of desirable plants at the seedling stage will save time and costs for the breeding program. DNA markers also have the potential to improve the efficiency and precision of conventional breeding via marker-assisted selection (MAS), if they are associated with desirable agronomic characteristics (Collard and Mackill 2007).

Confirming the ploidy level of the different species and specific elite parents selected from regional field evaluations is also very important, as some of the breeding strategies will involve crossing tetraploids with diploids to produce triploids. Ploidy levels will be measured using a Flow Cytometer. Ploidy and genome size estimates for a few leucaena species have been conducted previously using a FACS flow cytometer (Govindarajulu et al. 2011a). The samples were chopped and stained with propidium iodine, and the species *Lactuca sativa* was used as a size reference. The flow cytometry was effective for the estimation of most diploid species, but failed for some species, including *L. esculenta*, *L. pulverulenta*, *L. retusa* and *L. greggii*, that routinely released excessive mucilage upon homogenization (Govindarajulu et al. 2011a). Therefore, the estimates for representatives per species remain unresolved. Recently, a real-time quantitative PCR-based method for the estimation of genome sizes has been developed, based on the absolute quantification of genetic elements in a known amount (mass) of genomic DNA (Wilhelm et al. 2003). The estimation requires specific primer sets that amplify a single copy gene for each species, which would be available from the generated Indel/SNP database and the draft genome of *L. trichandra*. The real-time PCR-based method is a useful tool for the analyses of large numbers of species, individuals and tissues to investigate the changes in leucaena genome size during phylogenesis and is an effective alternative for samples that cannot be analyzed by flow cytometry.

Breeding strategies

To breed sterile leucaena, we are proposing the following strategies by ploidy level:

Tetraploids

We propose to work with *L. leucocephala* to develop a non-flowering cultivar. The availability of extensive genomic resources for some legume species such as pea (*Pisum sativum*) and soybean (*Glycine max*), and well-documented genetic synteny has enabled a comprehensive inventory of genes potentially relevant for flowering behavior, with specific roles in light perception, photoperiod response, signal integration and inflorescence development (Weller and Ortega 2015). The flowering genes with known functions in legumes and other plant species and a reference

genome of *L. trichandra* would contribute to the gene prediction and identification through homologous alignment. Thus, the finding would shed light on the manipulation of candidate genes for development of non-flowering leucaena lines. New genome-editing biotechnologies, including the clustered regularly interspaced short palindromic repeat (CRISPR), that allows breeders to target specific locations in the genome, hold great potential to speed up crop innovation. In 2018, the Australian Office of Gene Technology Regulator (OGTR) review proposed that organisms modified using site-directed nucleases, without templates to guide genome repair (i.e. SDN-1), would not be regulated as GMOs. The prospect of rapid and efficient genome editing raises concerns related to off-target effects; therefore researchers have engineered changes to the CRISPR-Cas9 genome-editing system that significantly cut down on "off-target" editing errors (Slaymaker et al. 2016). The 'enhanced specificity' SpCas9 variants could be useful for precision plant breeding that requires a high level of specificity. Additionally, the newly emerged base editing technology has been used for point mutation repair. Together with previously described base editors such as BE4, Targeted-AID and dCpf1-BE, adenine base editors (ABEs) greatly expand the scope of base editing that enables the programmable installation of all 4 transitions (C→T, A→G, T→C and G→A) in genomic sequences (Gaudelli et al. 2017). If using the reference genome of *L. trichandra* does not yield the desired outcome, we will endeavor to sequence the genome of the allo-tetraploid *L. leucocephala*.

Another potential use for gene-editing technology is to develop male- and/or female-sterile *L. leucocephala*. These gene-edited lines will be useful for producing commercial hybrid seeds with the added benefit of exploiting hybrid vigor as has been extensively used in crops (Horner and Palmer 1995; Saxena et al. 2010; Kim and Zhang 2018).

The CRISPR technology is a new frontier for crop improvement and would serve as a proof-of-concept study on generation of non-flowering leucaena lines and/or male/female genic sterile lines to produce hybrids.

Triploids

The strategy for producing triploids is to cross tetraploid species (*L. leucocephala* and/or *L. diversifolia*) with diploid species that have good forage attributes such as *L. collinsii*, *L. macrophylla*, *L. shannonii* and others.

The main tetraploid species being utilized in the crosses to generate triploids are *L. leucocephala*, *L. diversifolia* and *L. confertiflora. Leucaena leucocephala* is the main commercial species with long-term demonstrated forage attributes (Brewbaker 2016). *Leucaena diversifolia* is a very productive plant with slightly lower forage quality than *L. leucocephala* (Hughes 1998), but has already been used as a forage plant (Jones et al. 1998; Sotelo 2017). Jones et al. (1998) evaluated the animal production from *L. diversifolia* and *L. leucocephala* for 192 days at Landsdown (North Queensland, Australia). The mean liveweight gain from *L. diversifolia* CPI 33820 (539 g/d) was not significantly different from that for *L. leucocephala* cv. Tarramba (664 g/d) though significantly lower than for *L. leucocephala* cv. Cunningham (723 g/d). In a grazing experiment, at the International Center for Tropical Agriculture (CIAT), the production of a pure grass (*Brachiaria* hybrid cv. Cayman) was compared with that of a mix of the same grass and *L. diversifolia* (2,000 plants/ha). Beef production for the grass only treatment was 227 kg/ha in 207 days, while the mix with *L. diversifolia* produced 552 kg/ha in 207 days (Sotelo 2017).

Leucaena confertiflora is a multi-stemmed tree with maximum height of 4 m, highly resistant to psyllid (*Heteropsylla cubana*) and possibly cold-tolerant as well (Hughes 1998).

Diploid parents can be utilized directly or following interspecific crosses between diploid species to combine the positive attributes of different diploid species (Table 1). The diploid or interspecific diploid hybrid can then be crossed with a tetraploid to generate a triploid hybrid with combined attributes of 2 or 3 *Leucaena* species (Table 2).

Table 2. Interspecific crosses conducted in Western Australia during 2018 and 2019 within the *Leucaena* genus.

Female parent	Male parent
Diploid	
L. pulverulenta	*L. collinsii*
L. retusa	*L. collinsii*
L. shannonii	*L. lanceolata*
L. lanceolata	*L. shannonii*
Triploid	
L. pulverulenta	*L. diversifolia*
L. trichandra	*L. diversifolia*
L. retusa	*L. diversifolia*
L. shannonii	*L. diversifolia*
L. shannonii	*L. leucocephala*
L. diversifolia	*L. collinsii*
L. diversifolia	*L. macrophylla*
L. pulverulenta	*L. leucocephala*
Tetraploid	
L. pallida	*L. leucocephala*
L. diversifolia	*L. leucocephala*

Species and individuals within species will be selected according to their performance at 3 Western Australian field sites located in Carnarvon, Broome and Kununurra

representing the target soils and climate. More than 200 accessions from 15 leucaena species have been established as spaced plants in 2018 at each of these locations and will be evaluated for 3–4 years.

Some crosses are more effective in one direction than in the reciprocal direction and using the tetraploid parent as a female is usually more effective (Sorensson and Brewbaker 1994). We have observed in some cross combinations that, when *L. collinsii* was used as the female parent, the resulting hybrid pods contained flat and aborted seed. One way forward is to develop an embryo-rescue protocol to rescue the hybrid embryos or, alternatively, do reciprocal crosses with *L. collinsii* as the male parent to obtain the required hybrid embryos.

Embryo rescue

Embryo rescue is an in vitro technique that aids in the development of weak or immature hybrid embryos that may not survive and develop in vivo into viable seeds and plants. It has been widely used to prevent embryo abortion caused by interspecific incompatibility between the genomes of male and female parents resulting in improper endosperm development and hybrid embryo death (Shen et al. 2011). Embryo rescue involves excising the immature embryos and placing them on nutrient media under sterile conditions for growth and development. Embryo-rescue technology is a valuable tool that can be used for producing seed from interspecific crosses that are not fully compatible. It can also be used to decrease duration of each breeding cycle in compatible crosses. Leucaena seed development takes about 4 months from pollination to seed harvest (Real unpublished data). If this seed-filling phase can be reduced by embryo rescue and in vitro germination, it would accelerate the breeding cycle and allow us to process more than one generation per year (Castello et al. 2015; Pazos-Navarro et al. 2017). We aim to pinpoint the exact time of embryo physiological maturity so we can utilize this technique in leucaena breeding.

Propagation of sterile leucaena

The tetraploid non-flowering *L. leucocephala* will require vegetative propagation, while triploids can be established by seeds or by vegetative propagation.

Vegetative propagation

In vitro tissue culture and plant regeneration systems have been established previously for the *L. leucocephala* cultivar K636 (cv. Tarramba). Saafi and Borthakur (2002) reported that green shoots were generated from friable calli derived from cotyledon explants. Shaik et al. (2009) further optimized the system using cotyledonary nodes as explants with a focus on the selection and concentration of auxins and cytokinins in the medium. The tissue culture system facilitated the clonal propagation of tetraploid *L. leucocephala* and has the potential to be adapted for other species. Recently, an *Agrobacterium*-mediated transformation protocol to produce transgenic leucaena plants using immature zygotic embryo segments of green seeds as explant material has been developed (Jube and Borthakur 2009).

In order to successfully produce single-cross hybrids in self-incompatible (SI) crops, breeders must be able to generate homogeneous and homozygous parental inbred lines to produce the hybrid. The generation of these inbred lines is impossible in the case of SI. However, the use of doubled haploid technology offers opportunity to develop homozygous lines by generating plants directly from haploid gametes, such as microspores. Following duplication of the haploid genome, or chromosome doubling, the resulting plants are fertile and 100% homozygous. The resulting doubled haploid plants have significant value for plant breeding and gene mapping. The technology has been used for the successful production of spring wheat and barley doubled haploids in Australia (Broughton et al. 2014). A literature review enabled us to identify some general similarities between the protocols regarding explants, medium compositions and culture conditions for haploid plant induction across the various legumes including soybean, chickpea (*Cicer arietinum*) and lupin (*Lupinus angustifolius*) (Croser et al. 2006). The haploid research already conducted in species of the Fabaceae family provides hope for further developments for leucaena in the future.

Seed production

Triploid seed production orchards can be established with alternate rows of the selected tetraploid parent and rows of the diploid parent. The diploid parent has to be of a single genotype to exploit the gametophytic self-incompatibility system (Brewbaker 1982) and to achieve self-sterility of the diploid trees. Single genotypes of the diploid parent can be planted via vegetative propagation or by seed. To sow seed, doubled haploid plants will need to be produced and crossed to have a single genotype as follows: $S_1S_1 \times S_2S_2 = S_1S_2$ that can be sown as seed, while producing sterile progenies.

Seeds harvested from the single-genotype self-sterile diploid trees can be produced only by insect pollination with the tetraploid parent. All seed harvested from the diploid rows will be triploid seed. Seeds from the tetraploid trees will be mainly self-pollinated and these seeds would not be harvested.

Conclusions

Strategies to breed sterile leucaena for Western Australia include plant breeding and biotechnology tools to generate sterile lines at both the tetraploid and triploid ploidy levels.

For tetraploids, the main target species is the commercial *L. leucocephala* that is well known for its potential as a high-quality, productive and persistent forage. Gene editing technologies (CRISPR) will be utilized to edit out flowering genes and develop a non-flowering *L. leucocephala* and/or create male/female genic sterile lines of *L. leucocephala*.

For triploids, the strategy is to cross tetraploid species (*L. leucocephala* and/or *L. diversifolia*) with diploid species that have good forage attributes, such as *L. collinsii*, *L. macrophylla*, *L. shannonii* and others. Several of these triploid crosses have already been created and will be evaluated in the Kimberley and Pilbara regions of Western Australia for their agronomic performance and sterility.

Successful production of sterile populations will enable the benefits of sowing leucaena on pastoral leases in the Kimberley and Pilbara areas to be realized without concerns about the plants becoming another environmental weed like prickly acacia.

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Vegetative and micropropagation of leucaena

Propagación vegetativa y micropropagación de leucaena

TRAVIS IDOL, ADEL YOUKHANA AND RENIER PAUL SANTIAGO

Department of Natural Resources and Environmental Management, University of Hawaii at Manoa, Honolulu, HI, USA.
manoa.hawaii.edu

Abstract

To effectively utilize sterile hybrids of leucaena, efficient protocols for vegetative propagation are needed that meet different user requirements and capabilities. We developed and compared methods for propagating several sterile hybrids of leucaena and compared them with each other and with propagation via seeds for variety K636. Methods included air-layers, rooted cuttings, grafting and tissue culture (micropropagation). All methods required 14–20 weeks from generation of new shoots on the stock plant to production of rooted plantlets ready to outplant as compared with 6–8 weeks for seedlings of K636. Successful rooting was highest for air-layers and rooted cuttings. Grafting had lower success owing to a higher skill requirement for the propagator. Tissue culture showed promise, but use of field-grown material was limited by microbial contamination of propagation media. Rooted cuttings are the best option presently for operational-scale propagation, but the method requires a mist system or a carefully controlled non-mist environment. If an effective method can be developed, grafting of young shoots onto a seedling rootstock is an alternative that retains the advantages of a seedling tap root and requires fewer resources than rooted cuttings or tissue culture. In the case of grafting consequences of eventual resprouting of the rootstock deserve attention.

Keywords: Grafting, rooted cuttings, sterile hybrids, tissue culture.

Resumen

Para poder utilizar los híbridos estériles de leucaena en forma efectiva, se necesitan protocolos eficientes para la propagación vegetativa que se ajusten a las diferentes necesidades y capacidades de los usuarios. En este estudio desarrollamos y comparamos métodos para propagar varios híbridos estériles de leucaena y los comparamos entre sí y con la propagación a través de semillas de la variedad K636. Los métodos incluyeron acodo aéreo, enraizamiento de estacas, injertos y cultivo de tejidos (micropropagación). Todos los métodos requirieron entre 14 y 20 semanas, desde la generación de nuevos brotes de las plantas madre hasta la producción de plántulas enraizadas listas para ser trasplantadas, en comparación con 6–8 semanas para las plántulas de K636 provenientes de semillas. El enraizamiento más exitoso fue mediante acodo y enraizamiento de estacas. El injerto fue menos exitoso y requirió de una mayor especialización por parte del propagador. El método de cultivo de tejido demostró ser promisorio, pero la contaminación de los medios de propagación por microbios resultó ser una limitante. La técnica de esquejes enraizados es actualmente la mejor opción para la propagación a escala operativa, pero requiere de un cuidadoso control del ambiente, por ejemplo mediante un sistema de humidificación. Si se logra desarrollar una técnica efectiva, el injerto de brotes jóvenes usando como pie de injerto plántulas que provienen de semilla, es una alternativa que mantiene las ventajas de la raíz pivotante de la plántula y requiere menos recursos que el enraizamiento de estacas o el cultivo de tejido. En el caso de injertos las consecuencias de eventuales rebrotes desde las plantas madre merecen atención.

Palabras clave: Cultivos de tejido, enraizamiento de estacas, híbridos estériles, injertos.

Correspondence: Travis Idol, Department of Natural Resources and Environmental Management, University of Hawaii-Manoa, 1910 East West Road, Honolulu, HI 96822, USA.
Email: idol@hawaii.edu

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Introduction

Concern about the invasive potential of trees in the *Leucaena* genus has led to a desire to produce and plant sterile hybrids or varieties. Some of the interspecific crosses between diploid and tetraploid species have resulted in sterile or low-fertility triploid offspring (Sorensson and Brewbaker 1994; Brewbaker 2016). This provides opportunities to breed for sterility as well as providing suitable characteristics for a range of environments and end-product uses. As yet production of reliably sterile and phenotypically uniform or predictable F1 hybrid seed for large-scale outplanting has not been achieved. Alternatively, sterile plants can be obtained from physical or chemical mutagenesis (Oladosu et al. 2016). Thus, exploration and evaluation of vegetative propagation are needed, especially for sterile mutations of leucaena with important agronomic attributes, such as high biomass.

There are published studies describing successful propagation of leucaena via rooted cuttings (Hu and Chih Cheng 1981; Puri and Shamet 1988; Dick et al. 1998; Shi and Brewbaker 2006), grafting (Bray and Fulloon 1987; Brennan and Mudge 1998; Brewbaker 1988) and tissue culture or micropropagation (Dhawan and Bhojwani 1985; Puthur et al. 1998; Saafi and Borthakur 2002; Pal et al. 2012). Anecdotal reports indicate direct-planting of 2-m stakes may also be successful if harvested from saplings (Dahlanuddin pers. comm.; P. Larsen pers. comm.). Accessible guidebooks exist for setting up vegetative propagation systems for tropical woody plants that require relatively low sophistication or infrastructure (Longman 1993; Leakey 2012). Since each propagation method requires different levels of skill, labor, facilities and other resources and can generate different numbers of plants over time and space, there is a need for side-by-side comparisons to evaluate tradeoffs and make recommendations for specific contexts and end-uses. Addressing these needs was the objective of our study.

Materials and Methods

We compared several vegetative propagation methods, including air-layers (marcots), rooted cuttings, grafting and tissue culture (micropropagation) utilizing several self-sterile or fully sterile hybrids of leucaena and the cultivar K636 of *L. leucocephala* ssp. *glabrata*, as summarized in Table 1. Stock plants were field-grown clones of selected hybrids. Plants were pollarded to 1 m in height and allowed to resprout. New shoots were harvested for vegetative propagation when they were the required size for each method.

Seedlings

Seeds of *L. leucocepaha* ssp. *glabrata*, var. K636, were scarified in concentrated sulfuric acid for 15 min, rinsed in distilled water and germinated in 410 mL containers ('D25l small deepots', Stuewe & Sons, Tangent, OR, USA) filled with a commercial peat:perlite:vermiculite mixture ('Pro-Mix HP', Stuewe & Sons, Tangent, OR, USA). After germination, seedlings were placed in a secondary container and subjected to subirrigation twice daily to maintain adequate media water content. They were fertilized weekly using a liquid fertilizer mixture that was adjusted exponentially to match plant growth rates, as described in Idol and Diarra (2016).

Table 1. *Leucaena* varieties used for vegetative propagation.

Variety	Parent species	Fertility	Propagation methods
K636	*Leucaena leucocephala*[1]	Fertile	Cuttings, grafting, tissue culture
KX2	*L. leucocephala* × *L. pallida*	Self-sterile	Air-layers, cuttings, tissue culture
KX4	*L. leucocephala* × *L. esculenta*	Sterile	Air-layers, cuttings, grafting, tissue culture
KX5	*L. diversifolia* × *L. pulverulenta* (or *L. trichandra*)	Sterile	Air-layers, cuttings, tissue culture
	L. diversifolia K156 × *L. leucocephala* K500	Sterile	Air-layers
	L. leucocephala K8 × *L. trichandra* K738	Sterile	Air-layers
	L. macrophylla K158 × *L. lanceolata* S[2] K393		Air-layers
	L. diversifolia K156 × *L. pallida* K376	Fertile	Air-layers
	L. diversifolia K11 × *L. leucocephala* K8	Fertile	Air-layers
	L. pulverulenta K19 × *L. leucocephala* K8	Partially fertile	Air-layers
	L. collinsii K185 × *L. lanceolata* K264	Fertile	Air-layers
	L. lanceolata K10 × *L. lanceolata* S K393	Fertile	Air-layers
	L. diversifolia K156 × *L. lanceolata* S K393	Fertile	Air-layers

[1]All *L. leucocephala* varieties are of the subspecies *glabrata.*
[2]var. *sousae*, sometimes classified as *L. cruziana.*

Air-layering

We used ~3-month-old shoots to test air-layering. Vertical stems 4–7 cm in diameter were girdled twice ~5 cm apart and the bark removed. Stems were girdled only during periods of adequate soil moisture to ensure sufficient sap flow and ease of bark removal. The cambium tissue near the upper girdle was treated with a commercial rooting hormone (Clonex gel, 0.31% indole-3-butyric acid, Growth Technology Ltd), wrapped in moist clean sphagnum moss, and covered with clear food-grade plastic wrap. Air-layers were checked for the presence of roots 3–8 weeks after girdling. Girdled stems were vulnerable to breaking due to high or gusting winds.

Rooted stem cuttings

We followed the general protocol of Shi and Brewbaker (2006) to evaluate the success of rooted cuttings. Shoots were 3–6-weeks-old when harvested. The first 2-node stem section with a fully formed leaf was harvested. We tested 2 rooting hormone concentrations and exposure times with variety KX4. The rooting hormone selected was a commercial liquid concentrate (Dip-N-Grow) that contains 1% indole-3-butyric acid (IBA) and 0.5% naphthalene acetic acid (NAA). The concentrations selected were 1,500 ppm (1,000 ppm IBA, 500 ppm NAA), 3,000 ppm (2,000 ppm IBA, 1,000 ppm NAA) and a control of distilled water. The freshly cut surface of the stem was then placed in the hormone solution for either 5 seconds or 5 minutes. Three rounds of cuttings were taken from the same clone of KX4 and the data aggregated to account for the effects of seasonality or repeatability on rooting success.

Once an optimal rooting hormone concentration and exposure time were determined, based on the first round of cuttings, we performed a second test to evaluate the effects on rooting success of position on the stem. Three consecutive 2-node cuttings were taken of variety KX4 and KX5, with the first cutting taken as described above. Three rounds of cuttings were taken to evaluate the repeatability of the results.

Finally, we tested the genetic variability of rooting success among 3 clones each of KX2 and KX5, both of which showed low rooting success in the study of Shi and Brewbaker (2006). The first 2-node cutting was used and subjected to the optimal rooting hormone concentration and exposure time. Three rounds of cuttings were taken to evaluate the repeatability of the results.

After exposure to rooting hormone, cuttings were placed in 107 mL conical containers ('Ray Leach Cone-tainers', Stuewe & Sons) filled with a 2:1 mixture by volume of vermiculite:peat moss. The cuttings were placed in a glasshouse with 50% shade cover and misted using aerosolizing mist nozzles ('Arizona Mist', Orbit Irrigation Products, North Salt Lake, UT, USA) set to deliver 15 seconds of misting spray every 5 minutes from 07:00 h to 19:00 h daily. Rooted plants were transplanted into 410 mL Deepots and treated as seedlings until they reached outplanting size.

Grafting

We attempted grafting of leucaena using 2 different-sized stems. For the first group, we used non-lignified shoots that were ~2 mm in diameter. For the second group, we used semi-woody shoots that were 4–6 mm in diameter. In both cases, we used seedlings of variety K636 as the rootstock, and all leaves were removed from the scion. The diameter of the rootstock was matched as closely as possible to the diameter of the scion. For the first group (non-lignified shoots), we used a top-wedge graft to join the scion and rootstock and the stems were cut using a single-edge razor blade. For the second group (semi-woody shoots), we used 2 graft types. In the first round, scions of K636 and KX4 were grafted onto K636 seedling rootstocks using a top-wedge graft as was done with the smaller stems. In subsequent rounds, we grafted K636, KX2, KX4 and KX5 onto K636 rootstocks using a saddle graft created with a grafting tool that had a curved blade to create complementary cuts in the scion and rootstock. The graft union was wrapped in Parafilm and the grafted plant was covered with a polyethylene bag and placed in a controlled indoor environment with temperature ~25 ^{o}C and an artificial light source set to 12 h of simulated daylight (~240 μmol/m^2/s of photosynthetically active radiation) in each 24 h period. Ten days later, the bags were removed and plants were moved into a mist environment as described for the rooted stem cuttings until new shoot growth on the scion was deemed vigorous enough that the graft union was successful and the plant could be transferred into the subirrigation system.

Tissue culture

We carried out a preliminary investigation of micro-propagation to test the viability of vegetative material taken from field- and nursery-grown plants of sterile hybrids. Our starting material was excised stem sections containing an axillary bud from the first 6 nodes on green wood shoots that were 3–4 weeks of age (i.e. the same age and size as those used for cuttings). Excised stem sections were surface-sterilized by placing them in a bleach solution for 10 min. Afterwards, they were rinsed in distilled water and placed on an agar medium in a sterile

clear plastic container that was sealed and placed in a controlled environment with artificial lighting (12 h/d) and temperature ~25 ºC. Plants were checked every few days for signs of microbial contamination, general health and the presence of new shoot or root growth.

Comparison of propagation techniques

Since KX4 was the most commonly used sterile hybrid for vegetative propagation, we selected it for comparison of the techniques according to several criteria, including:

1. the number of rooted plantlets that survived to outplanting size per field-grown stock plant;
2. the time taken from stumping or pollarding the stock plant until the plantlets reached outplanting size;
3. the approximate labor time required to generate plantlets and the labor skills required; and
4. the resources or facilities required to successfully carry out each method.

Data analysis

For rooted stem cuttings differences in rooting success of cuttings based on rooting hormone concentration, exposure time and stem position were analyzed using analysis of variance (ANOVA). Main effects were analyzed separately, i.e. we ignored the interaction effects. For stem position, only a single main effect was analyzed. Tukey's honest significant difference test was used to compare main effect means among treatment levels. For comparison of size between cuttings vs. grafted plantlets, we used a t-test assuming unequal variances.

Results and Discussion

Air-layers

Air-layers were highly successful (>90%) under conditions of adequate soil moisture and sap flow, but girdled stems were vulnerable to stem breakage owing to high wind gusts. Losses varied by stock plant and season but could be as high as one-third of stems over 6 weeks. Owing to their large size and vigorous growth after harvest, air-layers are suitable for direct planting in the field. They quickly outgrow containers if potted and used as stock plants in the nursery. Wind protection of air-layered stems is highly recommended.

We did not rigorously test direct outplanting of stakes in the ground, a common propagation practice with another widespread multipurpose legume tree, *Gliricidia sepium* (Simons and Stewart 1998). We know of only one published study, which is preliminary in nature (Duguma 1988); it reports resprouting of above-ground shoots but not direct evidence of rooting or long-term survival and growth. Anecdotal reports of success with stakes taken from saplings are promising, but it cannot be assumed stump sprouts from older trees will demonstrate similar success. We explored the viability of this technique with five 3–6-month-old stump resprouts of seedless hybrids that are 20–25 years of age. Harvested stems were planted 40 cm deep in moist soil, but none of the stems produced roots or persistent shoots after 3 months.

Rooted stem cuttings

Rooting success of KX4 stem cuttings was highest at 1,500 ppm of rooting hormone concentration (Table 2) and there was no effect of exposure time. Success for control plants was <20%, averaged over 3 rounds of cuttings. The second stem position had the highest rooting success for both KX4 and KX5 (Table 3). Variety KX5 showed significant difference by clone in rooting success, but variety KX2 did not. Shi and Brewbaker (2006) had much lower success with these hybrids under standard propagation conditions. Only with the addition of an etiolation treatment of the stock plant did they achieve success rates >50%. As in their study, rooting success for cuttings taken and propagated during winter was much lower than in summer (data not shown).

Table 2. Rooting success (frequency, %) of KX4 stem cuttings at different rooting hormone concentrations and exposure times over 3 rounds of cuttings.

Hormone concentration (ppm)	Exposure time	Round			Average
		1	2	3	
0		20	10	22	17B[1]
1,500	5 sec	100	90	90	93A
1,500	5 min	80	80	90	83A
Average					88a
3,000	5 sec	65	80	54	66A
3,000	5 min	55	70	61	62A
Average					64b

[1]Values for treatments followed by different upper-case letters and averages followed by different lower-case letters are significantly different (P<0.05).

Grafting

Grafting of the smaller stems (2–3 mm diameter) was unsuccessful, while grafting of the larger stems (4–6 mm diameter) with a top-wedge graft was more successful: 90% for K636 scion onto K636 rootstock and 60% for KX4 scion onto K636 rootstock. Grafting using a saddle graft created with the grafting tool was largely unsuccess-

ful (<10%). While the tool provided rapid and complementary cuts on the scion and rootstock, the exposed tissue of the scion was not held tightly together by the rootstock as it was in a top-wedge or veneer graft. The blade on the grafting tool was also not as sharp as a razor blade or grafting knife, so it may have crushed some of the cambium cells as it sliced through the stem.

Table 3. Rooting success (frequency, %) of KX4 and KX5 cuttings taken from different stem positions over 3 rounds of cuttings.

Variety	Round	Two-node position		
		First	Second	Third
KX4	1	10	90	70
KX4	2	30	70	60
KX4	3	10	90	50
Average		17C[1]	83A	60B
KX5	1	8	66	45
KX5	2	20	58	53
KX5	3	10	75	60
Average		13C	66A	53B

[1]Within varieties average values followed by different letters are different (P<0.05).

Tissue culture

Microbial contamination of growth media and excised nodes required an adjustment of the bleach solution and sterilization time to a final concentration of 50% bleach and 10 min exposure. In addition, the agar medium was supplemented with antibiotics (cefotaxime - 250 mg/L; carbenicillin - 100 mg/L; and rifampicin - 50 mg/L). Final contamination rates ranged from 15 to 25% for different hybrids. There were no differences by position along the stem. After 6 weeks, some plantlets initiated new shoot and root growth (Figure 1), demonstrating their viability for use in media supplemented with hormones to induce callous tissue and multiple stem formation as in Pal et al. (2012).

Comparison of propagation techniques

Table 4 lists the average number of successful plantlets per field-grown stock plant of KX4 for each technique and the average time required to produce a plantlet ready for outplanting. Rooted cuttings had the best combination of number of plantlets harvested per stock plant (2 cuttings per harvested stem), success rate and time to achieve outplanting size (16 weeks). Plant material was generally ready for harvest from the stock plant 3–4 weeks after stumping or pollarding. The time to produce roots was approximately 4 weeks after placement in the mist system. After transplanting, rooted cuttings grew to outplant size in 8–10 weeks. Ignoring stem breakage due to high winds, air-layers had the highest success rate but the fewest stems per stock plant that were appropriate for propagation. Stems used for air-layering were on average 12 weeks old, and root formation was usually vigorous within 4–6 weeks. Stock plants produced about half as

Figure 1. Examples of: (**a**) axillary bud stem sections used for micropropagation of sterile *Leucaena* hybrids; and (**b**) successful growth of new shoots and roots in sterile medium.

many suitable shoots for grafting as for rooted cuttings. However, this is based on a single point-in-time count, 4 weeks after pollarding. They also took longer to reach outplanting size (20 weeks).

Table 4. Number of plantlets per stock plant and time to outplanting for various vegetative propagation techniques. Seedlings of K636 included for comparison.

Type	Number harvested	Percent success	Outplant number	Weeks to outplant size
Seedlings (K636)	NA[1]	75[2]	NA	6
Air-layer	10	90	9	16
Cuttings[3]	120	72	86	16
Grafting	30	60	18	20
Tissue culture[4]	360	ND[1]	ND	20[5]

[1]NA = not applicable, ND = not determined; [2]Starting from seed; [3]Based on harvest of 4-week-old stems and use of 2 nodes per stem; [4]Based on harvest of 4-week-old stems and use of 6 nodes per stem; [5]Estimated using development and growth rates from Pal et al. (2012).

Assuming 6 usable nodes on 4-week-old stems, stock plants produced on average 360 suitable shoots for tissue culture. With a 15–25% rate of contamination, that would result in ~250–300 plantlets for multiple shoot and root induction. While we have not yet tested multiple shoot induction, if results from Pal et al. (2012) are used as a guide, we may expect ~200 rooted plantlets per stock plant that could be ready for outplanting within 20 weeks. Since micropropagation techniques can rely on repeated rounds of shoot induction from cultured plantlets, it requires only limited success from field- or nursery-grown stock plants initially to then scale-up production to whatever numbers are desired. The time to outplanting size would not be much different, since it takes only ~1 week for cut stems of field- or nursery-grown stock plants to break bud and produce new shoots.

The estimated labor requirements to propagate a batch of 100 plants to outplanting size varied from a low of 200 minutes for air-layers and cuttings to a high of 355 minutes for tissue culture (Table 5). The estimated time for 100 seedlings, by comparison, was 150 minutes. The distribution of labor among propagation activities also varied, based on the requirements of the methods. This also relates to the type of training and skills required, which would affect the total cost of production.

Grafted plantlets were significantly larger than rooted cuttings at outplanting for all measured variables and had a greater root:shoot ratio (Table 6). Seedling size at 10 weeks was larger than either grafts or cuttings at what we considered outplanting size. This was due to our desire to grow seedlings to a basal stem diameter of ~5 mm to use as the rootstock for grafting. Root mass and part of the shoot mass of grafts thus represent the contribution of the rootstock. Seedlings of K636 reached a comparable height as cuttings and grafts of KX4 in 6–7 weeks after germination (Figure 2).

Table 5. Labor requirements (min/100 plantlets) for various vegetative propagation techniques. Seedlings of K636 included for comparison.

Type	PP	MP	PT	T	PM	Total
Seedlings (K636)	20	60	10	0	60	150
Air-layer	0	0	200	0	0	200
Cuttings	30	60	20	30	60	200
Grafting	185	0	50	0	60	295
Tissue culture	25	200	40	30	60	355

PP = Plant preparation; MP = Media preparation; PT = Propagation treatment; T = Transplanting; PM = Plant maintenance.

Table 6. Comparison of size at outplanting for rooted cuttings vs. grafted plantlets of sterile *Leucaena* hybrid KX4. Height and number of leaves for seedlings of K636 included as a standard. R:S, root:shoot ratio ('shoot' includes mass of leaves and stem).

Measurement	Seedling (K636)	Cuttings (KX4)	Grafting (KX4-K636)
Height (cm)	74	32b[1]	48a
Stem diameter (mm)	4.9	ND[2]	ND
No. leaves	12.3	5.8b	7.2a
Leaf mass (g)		1.17b	1.53a
Stem mass (g)		0.49b	1.25a
Root mass (g)		0.36b	1.84a
R:S ratio		0.22	0.55

[1]Values followed by different letters are significantly different ($P<0.05$); [2]ND = not determined.

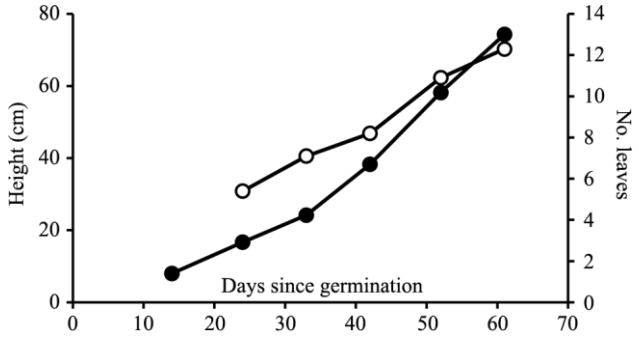

Figure 2. Growth of K636 seedlings 61 days after germination. Black circles = plant height; white circles = number of fully expanded leaves.

Recommendations

Each technique has its relative advantages and disadvantages and thus can be recommended for specific purposes and contexts. Air-layering requires the fewest resources and no nursery phase for production. It also requires the least amount of labor to propagate and no maintenance of the stem from the time of propagation treatment to harvest. The rooted stems are also much larger and thus establish and grow faster after outplanting than other propagation techniques, including seedlings. The main drawback is the relatively low number of stems per stock plant (~10). Air-layering of other woody species is commonly done on stems 1–2 cm in diameter, much smaller than we used in this study (4–6 cm). We have experimented with air-layering of smaller stems of KX4 and even setting multiple air-layers on a single stem, but success rates are generally lower. We hypothesize that the upper limit for successful air-layered stems that can be propagated at ground level is 20–30 per stock plant. If a producer starts with 20 trees as stock plants, then each round of propagation could produce 200–600 air-layers, provided there is appropriate wind protection to minimize stem breakage. This is adequate for small-scale utilization, e.g. as field borders, wind breaks, cut-and-carry fodder for small animal production or as overhead shade for crops in a field 1 hectare or less in size. Thus, it can be recommended for on-farm propagation for smallholder producers.

If direct outplanting of resprouting stems ~2 m in length proves viable, it would avoid the problem of wind breakage with air-layers, but it would not significantly alter the number of stems per stock plant. It could reduce the time to outplanting from 16 weeks to 12 weeks, but vigorous growth of planted stakes would still be delayed by 4–6 weeks as new roots would need time to form. Planting stakes deeper in the soil (50–100 cm) than air-layers (~15 cm) increases the labor for outplanting, especially in clayey or rocky soils. However, it does avoid competition for water with surrounding herbaceous vegetation. Our preliminary results suggest low viability of this technique, although it may differ by variety or hybrid.

Grafting can be used for slightly greater production levels (30 per stock plant), if the producer or a propagator has an adequate outdoor or indoor nursery and a technician with skill and experience in simple grafting techniques. Compatible rootstock-scion combinations need to be established to ensure graft unions are successful and that stem growth of the rootstock and scion are reasonably similar. Given the possibility that air-layering could generate 20–30 rooted stems per stock plant, grafting may seem unnecessary. However, the real advantage of grafting for leucaena is that a seedling rootstock can be used that has a healthy tap root to improve establishment, drought tolerance and recovery from regular browsing, pruning or other harvesting of the shoots. We have seen no decline in vigor of KX4 trees subject to pollarding 2–3 times per year over an 8 year period. However, an integrated grazing or cut-and-carry fodder production system in Hawaii would ideally harvest shoot material 6–8 times per year. The main disadvantage of grafting is the possibility of resprouting of the rootstock, which may represent either a suboptimal variety for forage production and quality or potentially a seeding variety that may be undesirable or restricted for outplanting in environ-mentally sensitive areas. The rootstock of Leucaena grafts will probably generate new shoots if the scion is browsed or pollarded, requiring attention from producers to prevent flowering and seeding of the rootstock. This is also a challenge in areas where seasonal frost may be significant enough to cause dieback of the stem below the graft union, i.e. close to the ground, as in subtropical areas of Australia or higher elevation sites in the tropics. Complete dieback of the scion would represent a failure of grafting as a propagation method.

Given the advantage of a tap root, the relatively low production level per stock plant is disappointing. Our early attempts to graft 2–3-week-old stems of either KX4 or K636 onto the rootstock of K636 seedlings failed. However, Brennan and Mudge (1998) did have success with single-bud splice grafting of shoots 2–3 mm diameter onto a similar-sized rootstock or via modified veneer graft onto rootstocks that were 5–15 mm in diameter. If production of grafts could be increased to the range of rooted cuttings (100–200 per stock plant), this could be a reasonable approach for commercial use beyond smallholder farms.

Rooted cuttings offer a balanced trade-off of rooted plantlets per stock plant, time to outplanting size and labor and skills requirements. The main drawback from our experience is the need for a mist system to protect the delicate leaflets from desiccation. Roger Leakey (2012, Ch. 7) describes and illustrates a standardized non-mist propagation chamber that can be constructed and used outdoors without the need for electricity or running water. It has been applied successfully for rooted cuttings of a number of tropical woody plants, but our attempts to replicate this in Hawaii with a variety of non-mist propagation chambers failed. We constructed a propagation chamber using Leakey's instructions and diagram, but even with overhead shade we were unable to keep the temperatures inside the chamber cool enough to sustain the cuttings and maintain high relative humidity

(>90%). We tried 2 separate indoor non-mist propagation chambers with artificial lighting and controlled temperatures (25–28 ºC) and a chamber placed inside a glasshouse to provide natural lighting. The chambers maintained adequate humidity with once- or twice-daily hand misting, but cuttings still failed to thrive or produce roots. Thus, we settled on a mist system following guidance from Shi and Brewbaker (2006).

One intriguing alternative for rooted cuttings was reported by Dick et al. (1998). They harvested single-node cuttings sequentially down the stem of 1-yr-old seedlings of *L. leucocephala*, dipped the cuttings in a rooting hormone (0.8% IBA powder), and placed them in a non-mist propagation chamber with a heated bed to maintain temperatures of 22–33 ºC. They found very high success (near 100%) for nodes 5–13 and success of >60% for 15 of 25 nodes evaluated. Stem diameter of nodes 5–25 averaged 4–7 mm, approximately the same as those we used for grafting. Our limited experimentation with stems of KX4 in this diameter range in a non-mist propagator was not successful. Seedling stems may retain a greater ability to produce roots from cuttings than stump sprouts, or there may be genetic differences between the hybrid KX4 and pure *L. leucocephala* that affect rooting success. This merits further study, since the ability to successfully propagate ~10 nodes per stem with a basal diameter of ~7 mm in a non-mist chamber could generate 200–300 plantlets per stock plant, more than we achieved with leafy stem cuttings in a mist environment. The tradeoff is leafy stem cuttings can be harvested, beginning 3 weeks after stumping or pollarding, whereas stems 5–7 mm in diameter with 15–25 nodes require at least 6–8 weeks of growth.

Assuming a combination of these 2 approaches could generate 200 rooted plantlets, this would produce ~4,000 plants from 20 field-grown stock plants every 6–8 weeks. This would be sufficient for outplanting a hectare of pasture in an integrated grazing system, given a within-row spacing of 50 cm and an inter-row spacing of 5 m. Since our stock plants were on a 2 m spacing, a single hectare could generate 500,000 or more rooted cuttings in a single propagation round, enough for 120 hectares of pasture.

Our work with micropropagation of sterile leucaena hybrids is too preliminary to reliably predict success rates. However, the advantage of tissue culture is that once success is achieved in vitro, successive rounds of propagation can be performed without having to rely on stock plants in the field or nursery. Thousands of plantlets used for propagation can be kept in a sterile laboratory room with supplemental lighting. However, this means producers must rely on commercial sources for planting material, and propagators must have adequate facilities, reliable infrastructure, dependable supply chains and sufficient technical expertise. Where such conditions prevail, even difficult-to-root varieties may be eventually brought into production and propagated at whatever scale is desired. The other advantage is that in vitro samples can be easily shipped wherever there are adequate facilities for local propagation. For introducing or expanding integrated grazing systems in an area at a scale of hundreds of hectares per year, this would be the recommended method for vegetative propagation.

Conclusions

Leucaena has been successfully propagated vegetatively using most of the common techniques, including air-layering, rooted cuttings, grafting and tissue culture. Application to sterile hybrids of leucaena will be necessary for their widespread use until or unless there is commercial-scale availability of seed of reliably sterile F1 hybrid varieties. Based on our experience with several sterile hybrids, we can recommend air-layering for on-farm production of stock plants or for smallholder farmers interested in using leucaena as wind breaks, crop shade or cut-and-carry fodder for small animal production. Where adequate nursery facilities are available, rooted cuttings can be generated from plantations of stock plants at sufficient scale to supply larger farms, including extensive integrated grazing systems. Grafting provides plantlets the advantage of a seedling tap root and does not require indoor propagation facilities, but this reduces the number of scions per stock plant compared with cuttings. Tissue culture is yet to be successfully demonstrated with vegetative material from sterile hybrids, but our preliminary work suggests future success is likely. This would allow for mass propagation needed to establish integrated grazing at the scale of thousands of hectares per year and for easy sharing of material among cooperators for local production. Our comparisons among techniques in terms of production rates and times and required labor, skill and facilities provide a useful guide for selecting and refining methods appropriate for the scale of desired production as well as the end-use for this multipurpose tree.

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(Note of the editors: All hyperlinks were verified 30 April 2019.)

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ILC2018 Poster and Producer paper*

Comparing the grazing productivity of 'Redlands' and 'Wondergraze' leucaena varieties

Comparando la productividad de las variedades de leucaena 'Redlands' y 'Wondergraze' bajo pastoreo

CRAIG LEMIN[1], JOE ROLFE[1], BERNIE ENGLISH[1], ROBERT CAIRD[1], EMMA BLACK[2], STEVEN DAYES[1], KENDRICK COX[1], LINDSEY PERRY[3], GREG BROWN[4] AND RONNIE & NADINE ATKINSON[5]

[1]*Queensland Department of Agriculture and Fisheries, Mareeba, QLD, Australia.* daf.qld.gov.au
[2]*Queensland Department of Agriculture and Fisheries, South Johnstone, QLD, Australia.* daf.qld.gov.au
[3]*Queensland Department of Agriculture and Fisheries, Cloncurry, QLD, Australia.* daf.qld.gov.au
[4]*Tolga, QLD, Australia*
[5]*Pinnarendi Station, Mt Garnet, QLD, Australia.* thebrickoven.com.au

Keywords: Cattle, liveweight gains, psyllids, Queensland Gulf Country, tree legumes.

Introduction

Leucaena is a rapid-growing, perennial, leguminous tree with the potential to sustainably intensify beef production in the northern rangelands of Australia (Harrison et al. 2015). Adoption of leucaena in the northern Australian beef industry has been slow, partly due to the prevalence of the sap-sucking leucaena psyllid (*Heteropsylla cubana)*. Recent efforts to develop new psyllid-resistant varieties have resulted in the release of cultivar Redlands, which has the potential to improve beef production in northern environments. A large-scale trial has been established to compare liveweight gains of cattle grazing Redlands, with that of the established cultivar Wondergraze in a psyllid-prone environment of north Queensland. This paper presents some preliminary results from the trial as the grazing phase commenced only in June 2018. Weight changes of successive groups of weaner steers (*Bos indicus* type) will be monitored over at least three 12 month grazing periods. Stocking rates in the first year are light to protect young leucaena plants, but will be increased in subsequent years when the leucaena is fully grown.

Materials and Methods

Preparation of trial site and psyllid monitoring

A 61 ha cleared trial site was selected at Pinnarendi Station (18.03849º S, 144.872453º E; 759 masl) on yellow to red-brown granite-derived soil with an average pH of 6.4 ± 0.07. Average annual rainfall is approx. 690 mm with the majority falling between November and April. Soil phosphorus and sulphur concentrations were low (5.1 ± 0.06 and 2.6 ± 0.15 mg/kg, respectively).

Prior to the 2016/2017 wet season, plant rows were set-out and prepared by strip cultivation. Superphosphate (9% P, 11% S) was applied at 300 kg/ha (27 kg P/ha; 33 kg S/ha) to a 1 m strip along plant rows before planting. Two leucaena treatments, cvv. Redlands and Wondergraze, were sown in an 8-paddock paired block design (Figure 1) in early 2017 during the wet season. After initial establishment, superphosphate was again applied at 280 kg/ha (25 kg P/ha; 31 kg S/ha) to a strip over the plant rows. Six months after planting, granulated sulphur (90% S) was applied over the leucaena rows at 160 kg/ha (144 kg S/ha) to provide sulphur for leucaena over the longer

Correspondence: C. Lemin, Queensland Department of Agriculture and Fisheries, 28 Peters St, Mareeba, QLD 4880, Australia.
Email: craig.lemin@daf.qld.gov.au

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Figure 1. Trial layout with 4 replicates of Redlands (R'lnds) and Wondergraze (W'grz) in 8 paddocks.

8 R'lnds 8.4 ha Water	7 W'grz 8.4 ha Water	Station Access Road	Yards/ Scales	6 W'grz 7.3 ha Water	5 R'lnds 7.3 ha Water	4 R'lnds 7.3 ha Water	3 W'grz 7.3 ha Water	2 R'lnds 7.3 ha Water	1 W'grz 7.3 ha Water
Laneway				Laneway					

Table 1. Pinnarendi actual rainfall for 2017 and 2018 and the long-term median from the closest weather station.

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
Pinnarendi 2017	235	131	126	15	16	5	2	8.5	0	80	5	40	663
Pinnarendi 2018	175	122	298	12	4	12	8	1	1	37	19	262	952
Long-term median[1]	152	191	98	25	16	11	6	0	0	12	50	118	679

[1]Long-term median from Meadowbank weather station (1956–2017; Bureau of Meteorology) located in the district.

term; superphosphate was broadcast across the whole site at 240 kg/ha (22 kg P/ha; 26 kg S/ha) to promote growth of the inter-row pasture. In February 2018, a contingency application of custom-blend fertilizer (12% N, 11% P, 10.5% S) was applied at 250 kg/ha (30 kg N/ha, 27.5 kg P/ha; 26 kg S/ha) over a 3 m strip along plant rows to address apparent suboptimal growth of leucaena during the 2017-2018 wet season.

Existing inter-row pasture species were retained and included Indian couch (*Bothriochloa pertusa*), Wynn cassia (*Chamaecrista rotundifolia*), Sabi grass (*Urochloa mosambicensis*) and *Stylosanthes* spp. The leucaena and pasture grew well, helped by useful late rainfall in May 2017 and unseasonal rainfall in October of the same year (Table 1, Figure 2).

Figure 2. Redlands leucaena in Sabi grass pasture at Pinnarendi after 2017 wet season.

The Pinnarendi site was deliberately selected in an environment where psyllids were known to be prevalent so that any productivity difference between Redlands and Wondergraze caused by psyllid damage could be expressed. No attempt is being made to control psyllids. A monitoring program using 9 sentinel plants per paddock (9 × 8 = 72 plants total) was set-up to record the degree of leaf damage caused by psyllid infestations. A modified rating scale (Wheeler 1988) was used, where 0 is no psyllids present and 9 is blackened stems with total leaf loss. Assessments were made on 9 occasions in 2017 and 4 in 2018.

Grazing Trial

The Queensland Department of Agriculture and Fisheries (DAF) Animal Ethics Committee approved animal handling and experimental procedures (SA 2017/12/628). Consistent groups of cattle have been grazing on the trial site since late June 2018 comprising 16 Droughtmaster (stabilized *Bos indicus* × *Bos taurus*) steers and 12 Brahman cross (*Bos indicus* × *Bos taurus*) steers. There are 4 treatment groups with 7 animals/group blocked according to breed (4 × Droughtmaster and 3 × Brahman cross per group) and weight (to achieve relatively similar initial total group weights). The groups were assigned at random to either Redlands or Wondergraze treatments. Sampling of biomass to estimate dry matter yields was done in the inter-row pasture in late July 2018. Leucaena biomass was sampled in paddocks 1–4 on 16 August prior to cattle entry and again after cattle were removed on 28 September. Paddocks 5 and 6 were also sampled in mid-September before cattle entry. A weather station was installed to monitor rainfall, temperature, wind speed and solar radiation. Electronic monitoring systems track tank water levels, while cameras remotely monitor watering points and cattle, while they are in proximity of the watering points.

Results

Establishment and psyllid observations

Growth of leucaena during the 2017-2018 wet season was suboptimal, despite earlier fertilizer applications. Potentially, this was attributable to nitrogen deficiency, caused by

poor root colonization with non-viable rhizobium inoculum (CB 3126) applied to seed before sowing. Overall, establishment of Redlands was worse than that of Wondergraze owing to differences in germination rates (30–45 vs. 80–90%, respectively). However, Redlands mostly compensated with increased growth so that final biomass was relatively uniform across all paddocks with the exception of Paddock 8, which has produced poorly.

Psyllids were active across the trial site from May to September 2017. Monitoring of incidence and damage showed that Wondergraze suffered significantly more damage than Redlands (Figure 3), but Wondergraze recovered quickly once psyllid pressure declined after September. Psyllid populations and damage were comparatively low during 2018 and are not reported. Inter-row pasture biomass (dry matter basis, DM; ± s.e.) was 6,020 ± 1,527 kg/ha across replicate paddocks at the site early in the dry season (late July 2018), comprising about 45% legume and 55% grass. Edible biomass (leaf and stem <5 mm diameter) of leucaena was only 65 ± 33 kg DM/ha in early July 2018 but had increased to 158 ± 51 kg DM/ha by late September in paddocks which had been spelled since late June. This was due to warming weather as there was no significant rain at the site since March 2018. Average daily liveweight gains (ADGs) have been determined for Redlands and Wondergraze treatments for the period of grazing from weighing events conducted in August and September 2018 (Table 2). These data are preliminary only and have not been analyzed for statistical significance. During this period, trial animals were also sporadically fed molasses (equating to about 2.5 MJ ME/hd/d) to accustom them to routine handling.

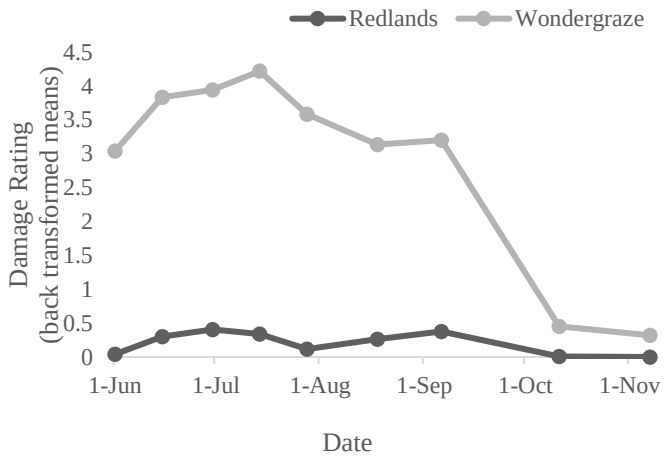

Figure 3. Psyllid incidence/damage in leucaena at Pinnarendi during 2017.

Table 2. Preliminary ADG data (± s.e.) for 28 steers grazing leucaena cvv. Redlands and Wondergraze at Pinnarendi Station, Mt Garnet district (28 June–20 September 2018).

	Average start weight (kg)	ADG (kg/hd/d)
28 Jun–7 Aug (40 days)		
Overall	231 ± 31	0.50 ± 0.21
Redlands	237 ± 35	0.47 ± 0.21
Wondergraze	225 ± 24	0.53 ± 0.20
7 Aug–20 Sep (44 days)		
Overall	248 ± 38	0.38 ± 0.18
Redlands	253 ± 43	0.32 ± 0.19
Wondergraze	244 ± 34	0.43 ± 0.15

Discussion and Conclusions

Preliminary results from the initial 3 months of grazing in the trial show cattle are gaining weight during the dry season with ADGs of about 0.4 kg. This is considerably higher than would be expected from native pastures at the same time of the year. While leucaena yield was low during the period and the grass-legume inter-row pasture will have contributed to this figure, leucaena was the only green feed available in the paddock and was high quality. Redlands was consumed readily by trial animals. To date, Wondergraze paddocks have produced slightly higher ADGs than those containing Redlands leucaena. However, the difference is not yet considered to be significant and the contribution of the inter-row pasture needs to be clarified. While psyllid resistance of Redlands was demonstrated during 2017, psyllid infestation during grazing in 2018 has been light and has not reduced growth of Wondergraze relative to Redlands. Performance of animals over the next 2–3 years is required to fully test the productivity of Redlands relative to Wondergraze grown within legume-grass pastures over a range of seasonal conditions.

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ILC2018 Poster and Producer paper*

‘Redlands for Regions’: Producer demonstration sites of psyllid-resistant leucaena across north Queensland

‘Redlands for Regions’: Un proyecto de sitios de demostración a nivel de productor de leucaena resistente a Heteropsylla cubana *en el norte de Queensland, Australia*

JOE ROLFE[1], BERNIE ENGLISH[1], CRAIG LEMIN[1], STUART BUCK[2], JIM FLETCHER[3], ROBERT CAIRD[1], EMMA BLACK[4], LINDSEY PERRY[5], BRON CHRISTENSEN[6] AND NIGEL TOMKINS[7]

[1]*Queensland Department of Agriculture and Fisheries, Mareeba, QLD, Australia.* daf.qld.gov.au
[2]*Queensland Department of Agriculture and Fisheries, Rockhampton, QLD, Australia.* daf.qld.gov.au
[3]*Queensland Department of Agriculture and Fisheries, Mackay, QLD, Australia.* daf.qld.gov.au
[4]*Queensland Department of Agriculture and Fisheries, South Johnstone, QLD, Australia.* daf.qld.gov.au
[5]*Queensland Department of Agriculture and Fisheries, Cloncurry, QLD, Australia.* daf.qld.gov.au
[6]*The Leucaena Network, Theodore, QLD, Australia.* leucaena.net
[7]*Meat and Livestock Australia, Brisbane, QLD, Australia.* mla.com.au

Keywords: Palatability, productivity, regional suitability, tree legumes.

Introduction

Leucaena, a tree legume with potential to greatly improve cattle performance, has not been readily adopted in northern Queensland primarily due to prevalence of the psyllid (*Heteropsylla cubana*) insect in higher rainfall zones. Psyllids reduce edible biomass in leaves by 40–52%, combined with a 46–83% reduction of stem yield (Bray and Woodroffe 1991). Losses to the Central Queensland beef industry due to psyllid impact on animal performance are estimated at $2 M per year (Mullen et al. 1998). Cultivar Redlands is a psyllid-resistant leucaena variety recently developed by Meat and Livestock Australia (MLA) and the University of Queensland. This new variety has the potential to lift productivity of cattle enterprises in the north. To accelerate early adoption and demonstrate benefits of the new variety to the grazing industry, the Redlands for Regions (R4R) project matched producer funds with PIFT-MDC (Producer Initiated Fast Track-MLA Donor Company) funding. The R4R project, led by The Leucaena Network (TLN), includes 7 trial sites in psyllid-prone areas with moderate to high rainfall from Mackay to the Atherton Tablelands in north Queensland. These sites will act as a platform for industry promotion and adoption of this promising new variety in accordance with TLN Code of Practice (CoP). The project supplied seed and technical assistance via Department of Agriculture and Fisheries (DAF) staff during the preparation and establishment phases to demonstrate best management practice for leucaena in these psyllid-vulnerable rainfall zones. This paper summarizes the extension processes employed during the project and highlights the challenges and successes at the project sites.

Planning and Site Selection

In May 2017, north Queensland-based DAF staff compiled a list of producers interested in establishing leucaena and the merits of each site based on location, soil types, expected psyllid pressure and the agronomic skills, confidence and capacity of the particular producers. In October 2017, producer agreements were finalized with 6 property owners (Table 1) with an additional property owner selected in December, independent of R4R funding.

The DAF team assisted MLA with engaging producer co-operators for the project. Once MLA had selected the sites, DAF provided technical and agronomic support including soil tests and interpretation, equipment requirements, fertilizer recommendations, seedbed preparation, planting and herbicide advice. Early development and

Correspondence: J. Rolfe, Queensland Department of Agriculture and Fisheries, Mareeba, QLD 4880, Australia.
Email: joe.rolfe@daf.qld.gov.au

*Poster presented at the International Leucaena Conference, 1–3 November 2018, Brisbane, Queensland, Australia.

Table 1. Names and location of 7 beef producers and their properties involved in the early pilot program for growing the Redlands leucaena variety in north Queensland.

Region	Property and Owner	Expected prevalence of psyllids
Mackay	Hazelwood, Mackay - Mark and Linda Degura	Moderate to high
	Mt. Spencer, Nebo - David Wright	Moderate to high
	Woonon, Sarina – Wayne and Scott Davis	Moderate to high
Townsville	Swans Lagoon, Millaroo - Peter Malpass	Moderate to high
	Four Mile, Woodstock - Gerard and Elizabeth Lyons	Moderate to high
Northern	Goshen, Mount Garnet - Brett Blennerhassett	Very high
	Quincan Springs, Peeramon - Peter and Colleen McLucas	Very high

establishment success were monitored at the respective sites where applicable, and local paddock walks conducted. Members of TLN and DAF coordinated 6 field days (total of 55 producers attended) on the northern sites to expose beef producers (R4R producers and the wider industry) to the latest leucaena establishment and production techniques. Field day topics included site selection, site preparation, planting, establishment and herbicide programs.

Materials, Methods and Results

Goshen (Brett and Theresa Blennerhassett)

The Goshen site consists of infertile red-earth soils. The Blennerhassetts purchased a heavy-duty Norseman twin-row leucaena planter with precision depth control (Figure 1) and leucaena was sown in February and March 2018 in twin rows (900 mm apart) with inter-row spacing of 10 m. Placing seed at 15–25 mm depth reduced time to emergence and improved overall establishment in comparison with planting at depths >30 mm under the same conditions. Approximately 56 ha of Redlands was successfully established at Goshen. Performance of this stand will be observed in comparison with a 40 ha stand of the psyllid-prone cultivar Cunningham, which pre-dates the Redlands planting.

Quincan Springs (Peter and Colleen McLucas)

This site on the Atherton Tablelands has deep, fertile red basalt soils. Redlands was sown into 32 ha (divided into 4 × 8 ha paddocks) in single rows with 15 m inter-row spacing in February 2018 using an adapted corn planter. Despite problematic seedbed preparation due to project delays and high residual organic matter levels, good establishment was achieved across the entire site. Weed control (tropical grasses and legumes plus broad-leaf weeds) was challenging at the site. Despite several frosts during June, leucaena was not affected. Stock were introduced to the site in August 2018 when leucaena plants were about 2 m tall (Figure 2).

Figure 1. The Norseman precision planter (left) and twin row Redlands leucaena seedlings (right) on Goshen.

Figure 2. Peter McLucas (Quincan Springs) and Bernie English (DAF) inspect leucaena seedlings (top) and cattle grazing the trial area in August 2018 (bottom).

Townsville sites

The soils at the Townsville site, Four Mile, are infertile, poorly structured yellow sandy-earths with low water-holding capacity and poor drainage. They were deemed unsuitable for leucaena establishment. Despite this, the Lyons family invested considerable effort in ground preparation and refining sowing techniques. Soils at the Swans Lagoon site were only marginally better and were also considered unsuitable for leucaena. About 30 ha of Redlands was sown at each site during February and March 2018 as conditions allowed but the unsuitable soils, plus hot, dry conditions and weed competition all contributed to poor establishment. When inspected in May 2018, leucaena was small and unthrifty at both sites and unlikely to survive.

Mackay sites

No attempt was made to establish leucaena at the Mackay sites in the 2017/18 growing season. Site preparation is underway currently at all sites and planting will take place over the 2018/19 spring-summer period.

- At Woonon, soil testing and interpretation have been completed across the 27 ha paddock selected for sowing with leucaena. Paddock clearing and initial cultivation have also been completed. A challenge with this site is high grass yields (>10,000 kg DM/ha).
- At Mount Spencer, the Wright family is being assisted by agricultural consultants Farmacist. In addition to soil sampling and analysis the 16 ha paddock was cleared of regrowth in 2017 and cultivated twice in late August 2018. Planting strips at 10 m spacing have been marked with a GPS and double-ripped. Paddock and soil variability will also be mapped using Electro-magnetic (EM) surveys prior to planting.
- At Hazlewood, an old sugarcane paddock was cultivated in March 2018 to incorporate trash from the previous cane crop. Soil samples have been collected and EM mapping data are available. Two additional cultivations were performed in May and the site was planted to pasture. Strips for planting leucaena rows were ripped at 10 m spacing using a GPS guidance system. Leucaena will be planted in the 2018/19 wet season in single rows with 10 m inter-rows.

Discussion and Conclusions

Under R4R, Redlands has been successfully established at both northern sites. At Goshen, planting of further areas with Redlands is planned for the 2018/19 growing season. The Townsville sites seem unsuitable for leucaena and would be more suited to development with improved pastures comprising a mix of grasses and legumes such as Rhodes (*Chloris gayana*), Keppel (*Bothriochloa pertusa*), Seca (*Stylosanthes scabra*) and Verano (*S. hamata*). Despite these recommendations, collaborators at both sites intend to plant more areas with Redlands in 2018/19. At Swans Lagoon, irrigated soils currently being trialled with Rhodes grass and *Desmanthus* sp. would be better suited to leucaena than the paddock previously used. DAF staff will continue to advise managers of Swans Lagoon to locate future leucaena plantings on these areas.

Learnings

Experiences from the project reinforce what is already known. Successful leucaena establishment is dependent on selection of appropriate soils/land types, good seedbed preparation, adequate soil moisture, good weed control, timely access to equipment and acquiring the necessary agronomic skills. Correct setting and control of planting depth were also of particular importance at the sites selected.

Future

While the R4R program is due to be finalized by March 2019, recommendations for future work include:

- Recording the performance of cattle grazing Redlands at the Mackay sites, assuming successful establishment during the 2018/19 wet season;
- Recording the performance of cattle grazing Redlands at Quincan Springs [to assess the cost: benefit of adding leucaena to highly productive grass-legume pastures on the Atherton Tablelands, which already achieve liveweight gains (LWGs) up to 250 kg/head/year]; and
- Comparing LWGs produced on Redlands with that on Cunningham at Goshen.

Such project activities would link closely with the Pinnarendi grazing trial (Mount Garnet), where productivity of Redlands is being compared with that of Wondergraze and initial grazing data indicate daily LWGs during the dry season of 0.4 kg/head. Continuing a series of trial and demonstration sites across north Queensland will expose beef producers to the practical challenges and production benefits of growing leucaena and sustainable management under the leucaena CoP.

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ILC2018 Keynote paper*

Establishment of leucaena in Australia

Establecimiento de leucaena en Australia

STUART BUCK[1], JOE ROLFE[2], CRAIG LEMIN[2] AND BERNIE ENGLISH[2]

[1]*Department of Agriculture and Fisheries, Rockhampton, QLD, Australia.* daf.qld.gov.au
[2]*Department of Agriculture and Fisheries, Mareeba, QLD, Australia.* daf.qld.gov.au

Abstract

Leucaena (*Leucaena leucocephala* ssp. *glabrata*) is a highly productive tropical perennial legume used primarily in extensive beef grazing systems across northern Australia. Its productivity provides substantial benefits to grazing businesses and economically significant areas of leucaena have been established in Queensland, with much smaller areas in both the Northern Territory and Western Australia. Specific environmental conditions (particularly soil type) and management practices are required to obtain reliable establishment and high productivity from leucaena-grass grazing systems. Significant research, development and extension have been undertaken in northern Australia, particularly in central Queensland, resulting in management packages which ensure establishment reliability and long-term productivity. However expansion into new areas can be constrained by regionally-specific establishment issues. Adaptation of known establishment and management practices together with research and development are required for leucaena-grass grazing systems in new regions.

Keywords: Planting, seed, tree legumes, tropical pastures.

Resumen

Leucaena (*Leucaena leucocephala* ssp. *glabrata*) es una leguminosa perenne tropical altamente productiva que se utiliza principalmente en sistemas extensivos de pastoreo de ganado de carne en todo el norte de Australia. Su productividad proporciona beneficios económicos sustanciales para los productores, y se han establecido significativas áreas de leucaena económicamente significantes en el estado de Queensland y en menor medida en los estados Northern Territory y Western Australia. Se requieren condiciones específicas tanto ambientales (particularmente respecto al tipo de suelo) como de prácticas de manejo para obtener un establecimiento confiable y una alta productividad de los sistemas de pastoreo con leucaena asociada con gramíneas. Se han llevado a cabo importantes actividades de investigación, desarrollo y extensión en el norte de Australia, particularmente en el centro del estado de Queensland, las cuales han resultado en paquetes tecnológicos que garantizan el establecimiento confiable y productividad a largo plazo. Sin embargo, la expansión a nuevas áreas puede verse limitada por problemas de establecimiento específicos de cada región. Se requiere que las prácticas de establecimiento y manejo conocidas sean adaptadas y acompañadas por actividades de investigación y desarrollo para los sistemas de pastoreo de leucaena-gramíneas en esas nuevas regiones.

Palabras clave: Árboles leguminosos, pastos tropicales, semilla, siembra.

Introduction

Leucaena is a highly productive tropical perennial legume which has been sown on many extensive beef grazing properties across northern Australia. When successfully established in 'rundown' (declining productivity due to the reduction of plant-available nutrients) grass-only sown pastures in tropical and subtropical environments, well-managed leucaena can improve both stocking rate and animal liveweight gain by up to 100% (Dalzell et al. 2006), providing significantly higher animal production per hectare per year (Bowen et al. 2018).

Correspondence: S.R. Buck, Department of Agriculture and Fisheries, Rockhampton, QLD 4700, Australia.
Email: stuart.buck@daf.qld.gov.au

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Despite these advantages only around 130,000 ha, i.e. about 0.5% of the potential area (Buck et al. 2019), has been sown across northern Australia. Early attempts at establishing leucaena often failed, typically due to graziers not following recommended cultural practices for reliable establishment (Lesleighter and Shelton 1986). In Australia, most cultivated leucaena is sown where suitable soils occur (Beutel et al. 2018), primarily under suboptimal climatic conditions of low and variable rainfall and cool winter temperatures, i.e. the subtropics (Middleton et al. 1995), that exacerbate the difficulties of establishment and management over time.

Significant research, development and extension have been conducted to overcome the establishment and management issues hampering adoption. This paper outlines the critical aspects for reliable establishment of leucaena across northern Australia.

Background of leucaena adoption and establishment in Australia

While a number of factors impeded the initial adoption of leucaena (Buck et al. 2019), unreliable establishment was a key reason (Lesleighter and Shelton 1986; Pratchett and Triglone 1989; Middleton et al 1995). Slow early growth of seedlings is a biological characteristic of the leucaena plant (Piggin et al. 1995), which increases vulnerability to environmental setbacks (Shelton and Jones 1995). However, establishment failures are often due to: a poor understanding of climatic and soil requirements and agronomic traits; the use of inappropriate cultural practices; and limited supply of high-quality seed (Lesleighter and Shelton 1986; Piggin et al. 1995; Shelton and Jones 1995; Middleton et al. 1995; Larsen et al. 1998). While knowledge of suitable climate and soil characteristics for leucaena were quickly determined (Gray 1968; Cooksley et al. 1988), the understanding of agronomic traits and implementation of best management practices took longer and was achieved only through the combined efforts of research workers, extension practitioners and graziers. Training courses were delivered in the early 2000s and a practical manual outlining effective establishment and management practices (Dalzell et al. 2006) was published in 2006 (Shelton and Dalzell 2007).

Site selection and layout for successful leucaena establishment in Australia

Climatic requirements

Leucaena is a tropical plant that grows best in warm-hot climates with mild winters and annual rainfall >600 mm. Temperature determines where leucaena should be established and how it is managed over the grazing year. Seed germination is highly influenced by temperature and soil temperature at planting should be at least 18 ºC (Dalzell et al. 2006). Leucaena growth also depends on temperature and typically slows or stops during the winter months, when annual average daily ambient temperatures fall below about 15 ºC (Cooksley 1986). Under irrigation in Western Australia, leucaena growth was depressed during June and July (winter) with average minimum temperatures of 14 ºC (Middleton et al. 1995). Frost can significantly limit growth with light frosts causing leaf drop, while stems usually survive. Heavy frosts can kill stems with subsequent regrowth occurring from the crown or larger surviving branches.

Leucaena is most productive in higher rainfall environments in the warmer subtropics and tropics, but psyllid infestations (*Heteropsylla cubana*) frequently, and sometimes severely, limit growth of susceptible cultivars (cvv. Peru, Cunningham, Tarramba and Wondergraze). The release of the highly psyllid-tolerant cultivar Redlands in 2017 should provide the opportunity for a further 1.2 million ha of previously unsuited coastal and northern Australian landscapes to be established to leucaena (Shelton and Dalzell 2007).

Soil requirements

Leucaena grows poorly in shallow and infertile soils, particularly those with chemical imbalances and soil structural issues (Cooksley et al. 1988) and should be sown into well-drained soils with high water holding capacity and fertility, i.e. arable soils. It has a high capacity to access soil water and nutrients from depth and can maintain growth during dry conditions when established in deep (>1 m) soils with high water holding capacity (loams - clays).

Leucaena does not grow well in acid soils and soil $pH_{1:5}$ below 5.5 restricts performance. While acidity in the top soil can be addressed economically with lime application(s), subsoil acidity is common in northern Australia and is difficult and costly to correct. Adequate on-going supplies of phosphorus, sulphur, potassium, calcium and zinc are critical for leucaena growth. While all can be supplied from fertilizer if the soil is deficient, correcting major deficiencies will significantly increase production costs and may reduce profitability.

Soils should be free-draining. Soils prone to prolonged waterlogging are unsuitable for leucaena, although mature plants are more tolerant than seedlings. Clay soils with high magnesium and/or sodium concentrations have poor soil structure and cause production limitations, so should be avoided. High levels in the topsoil reduce

seedling establishment, while high levels in the subsoil impede drainage and root penetration. In addition, on clay soils with high salt (chloride) levels in the subsoil, growth is limited through a direct toxic impact and/or restricting root penetration plus water and nutrient uptake. The key is to undertake a comprehensive soil test of both topsoil and subsoil prior to sowing new stands as an aide to decision making on paddock suitability and fertilizer requirements.

While leucaena performs best on deep, well-drained fertile soils, these soils are often located on low-lying parts of the landscape close to water courses. Establishing leucaena in these situations can pose a high risk in the form of unwanted leucaena (weed) spread if not managed appropriately. The Leucaena Network, a not-for-profit organization promoting the sustainable adoption of leucaena, has developed a Code of Practice that outlines where leucaena should be established and managed to maximize production while minimizing the environmental risks (Christensen 2019). Amongst other things, the Code of Practice states that leucaena should not be sown in areas close to where rivers, creeks and flood channels can disperse seed pods and seed.

Layout of leucaena plantations (row systems) in Australia

Conventionally, leucaena is sown in rows with native or sown grass pastures in the inter-row spaces. Initially, single rows were sown but to minimize gaps from failed establishment, twin rows ~1m apart are now commonly used in central and southern Queensland, while single rows are typical in north Queensland. It has also been suggested that double-row plantings restrict leucaena height due to competition between the rows, which increases accessibility to forage by stock (Dalzell et al. 2006). Double-row sowing may improve establishment uniformity in northern environments where adequate seedbed preparation, lighter soils and access to modern planters can be significant issues.

One of the most hotly debated topics at the International Leucaena Conference 2018, Brisbane, was the optimal inter-row width, i.e. between pairs of twin rows. Early plantings were based on narrow inter-row width of 1.5–5 m (Jones et al. 1982) but later plantings are more commonly at wider spacing of 6–10 m (Dalzell et al. 2006). This trend reflects the desire of some producers to maintain an adequate grass component in the diet of grazing animals relative to available leucaena biomass. Grass is a critical component of the leucaena-grass grazing system and can provide around 50% of the diet over the grazing year (Bowen et al. 2018). Grass also gives other benefits including: a feed supply during the drier and colder winter period when leucaena is less productive in tropical and subtropical regions; improved ground cover to increase water infiltration; reduced opportunities for weed colonization; an outlet for nitrogen fixed by leucaena to lift overall pasture productivity and encourage on-going fixation; and improved soil organic matter levels and carbon storage. Other reasons for widening inter-rows include lowering the seed cost per hectare, ease of mustering livestock and lower cultivation costs when sowing leucaena in fallowed strips. Some producers prefer wider inter-row spacing as it allows the operation of machinery between the rows to permit flexibility in terms of spraying inter-row weeds or trimming out-of-reach branches to improve forage utilization. Other producers require enough space for machinery to sow annual forage crops between the rows to improve overall forage production. On the other hand, since 100% leucaena is successfully fed to cattle in Southeast Asia (Dahlanuddin et al. 2014; 2019), some research workers and graziers argue that narrow inter-row spacing can be more productive. This topic warrants investigation.

The alignment of leucaena rows requires careful consideration. In general, rows should align with the direction of cattle movement for ease of mustering and, where possible, run across the slope to minimize erosion on sloping land. Aligning rows east-west to minimize shading of the inter-row grass pasture is being considered by graziers to maximize overall pasture production, although no research has been conducted into this aspect. The shading effect when rows are aligned north-south is less significant when wider inter-row spacings (>10 m) are used on soils of lower productivity. However when grown on productive soils at closer inter-row spacings (<8 m), the leucaena canopy can converge and substantially reduce grass growth, particularly when companion species are not shade-tolerant (Lemcke and Shotton 2018).

Best practice considerations for successful establishment in Australia

High rates of establishment failure have been linked to poor adherence to recommended seed preparation practices (Lesleighter and Shelton 1986) and poor weed control (Larsen et al. 1998). Graziers who do not follow recommended agronomic practices face significantly higher risks of establishment failure (Dalzell et al. 2006; Buck et al. 2012). A friable seedbed, high amount of stored soil moisture, effective pre- and post-sowing weed control, good quality seed, adequate planting rates, correct planting depth and good seed-soil contact are all critical for consistent leucaena germination and vigorous seedling growth. Seed should also be mechanically scarified and inoculated with the correct rhizobium.

Planting depth

Although leucaena has a relatively large seed (~22,000 seeds/kg), precision planting at depths ranging from 20 to 40 mm, depending on soil characteristics, is essential. Planting depths can be up to 40 mm in heavier, friable soils with good soil moisture but should be shallower in lighter soils. In more northern environments the risks associated with sowing into lighter soils include: greater evaporative potential leading to more rapid depletion of soil moisture; a propensity for soil surface sealing; and high probability of heavy rainfall after sowing washing soil onto the plant row (burying seedlings) and translocation of surface-applied pre-emergent herbicide, either reducing its effectiveness or making it toxic to the crop. Sowing at depths >25 mm in these soils, to access soil moisture at depth, generally leads to slow and poor emergence. The key to establishment success in these situations is timely and precise sowing (at depths of 20–25 mm) into good soil moisture with the reasonable assurance of imminent rainfall (within 5–7 days). In heavier soils, moist conditions for up to 7 days after sowing should ensure good germination and reliable emergence (Dalzell et al. 2006).

Ripping the plant row

Deep-ripping the soil where leucaena will be planted prior to sowing may improve water penetration and moisture storage during the fallow period, and promote a more vigorous root system after planting. One study recommends that decisions on ripping be based on the soil type to avoid unnecessary costs in undertaking this operation (Buck 2013). Ripping the soil along the plant row before sowing improved establishment (plant population) and growth (edible biomass) at 4 months after sowing on a non-cracking loam soil, whereas no benefits were measured on a cracking clay soil. Even though the benefits are short-lived on a responsive soil, techniques that improve the reliability of leucaena establishment are worth considering owing to the high cost of replanting. In north Queensland, basalt soils are suited to leucaena but typically contain many rocks. Deep ripping of these soils is required to develop a seedbed and allow the passage of heavy-duty planting machinery.

Planter technology

Leucaena is normally sown at 1–2 kg seed/ha depending on inter-row spacing (wider rows decrease seeding rate/ha), seed size (larger seed increases the seeding rate/ha) and seed viability (aim for >85% germination). Planters must have press-wheels behind the soil-opener (tyne or disc), ideally configured as a pair pressing from both sides of the plant line (not over the top) to carefully pack moist soil around the seed to maximize seed-soil contact. Planters fitted with water injection equipment are considered to significantly improve the reliability of nodulation by directly placing rhizobium into the seed furrow. This technique is now commonly used by contractors and experienced leucaena growers and is particularly beneficial when planting during hot conditions.

Weed control

As leucaena seedlings are slow to develop, weed control during establishment is critical. Water extraction by weeds and grasses in close proximity (<2 m) to the planted row can significantly inhibit growth of establishing leucaena seedlings. It is necessary to control weeds in this zone for up to 6 months post sowing (Dalzell et al. 2006; Peck et al. 2017). Traditionally weeds emerging after sowing have been controlled in part by inter-row (with tyned cultivators) and in-row (with Yetter™ wheels) cultivation. However, in recent years the availability of a residual herbicide, 700 g/kg Imazethapyr (trade names include Spinnaker, Impale, Amaze and Vezir), used under Australian Pesticides and Veterinary Medicines Authority (APVMA) permit no. PER82166, has revolutionized establishment success of leucaena through pre-emergent control of many broadleaf and grass species for up to 6 months. Larsen et al. (1998) suggested that consistently high establishment success rate (>90%) across the leucaena industry would be achievable only when effective and selective chemical weed control methods were available, and that time has now arrived.

Leucaena establishment in different regions of Australia

Central Queensland

The major region for leucaena production in Australia is the inland areas of central Queensland owing to the favorable climate, low psyllid incidence and availability of productive soils across large areas of cleared and readily-cultivated landscapes (Buck et al. 2019). One landholder in this area has 6,000 ha established to leucaena and sown grass pastures (Harris and Harris 2019). A history of cropping in the region has provided the combination of infrastructure, equipment and knowledge, which has enabled consistent and reliable establishment.

Based on advice to plant leucaena on high-quality soils, typically paddocks used for dryland crops or forages were initially sown. Following success in these situations, paddocks with a legacy of cultivation but having reverted to perennial grass-only pastures were also sown. In this situation leucaena provides significant production and economic benefits by increasing protein supply to grazing stock and adding soil nutrients to boost nitrogen-deficient grasses. Without legume inclusion, production from these grass-only pastures progressively declines over time as the supply of plant-available nitrogen also declines, commonly called pasture rundown (Peck et al. 2011). In these (existing) pasture situations, the following methods have been used successfully to establish leucaena: (i) Fully remove the existing pasture and fallow the whole paddock. This is the most appropriate method for pastures with severe rundown and/or pastures containing undesirable grass species, and provides the best opportunity for reliable and quick establishment. However fallow costs are significant, re-seeding of grasses is generally required and grazing must be withheld for a longer period of time. (ii) Prepare strips for sowing leucaena and retain the existing pasture in the inter-row spaces. This method is better suited to pastures that are still productive (i.e. low-moderate rundown) and contain desirable grass species. The strips need to be wide (~5 m) and either cultivated or sprayed to minimize competition for soil moisture between leucaena and inter-row grass pasture. With this technique, fallow costs are lower (around 50%), re-sowing of the grass is (generally) not required, and non-grazing periods are significantly reduced. Regardless of the method adopted, establishment success depends on: a fallow period of up to 12 months prior to sowing to store sufficient soil moisture to sustain the young plant (soil moisture profiles under pastures are typically very dry); a friable seedbed free of weeds; and the use of fertilizer to correct initial soil nutrient deficiencies and promote rapid establishment and initial growth.

Commonly, little or no fertilizer is applied to new or existing leucaena pastures (Radrizzani et al. 2010) and poor productivity from older leucaena-grass systems in paddocks previously used for grain or forage cropping is an emerging issue in central Queensland (Buck et al. 2019). It is likely that soil nutrient deficiency is a key cause of low biomass production and animal performance in many existing stands. This issue warrants investigation to identify and promote cost-effective fertilizer solutions.

Southern inland Queensland and New South Wales

Historically, inland southern Queensland from Wandoan to the New South Wales border was deemed too cold for acceptable leucaena growth (Lambert 2009). However over the last 10–15 years increasing areas are being sown to leucaena due to declining production from grass-only pastures, and issues associated with annual cropping systems including soil fertility decline and unreliable rainfall conditions (Lambert 2009; Emery and Sneath 2015). Establishment techniques used in southern Queensland generally mirror those used in central Queensland. Leucaena is commonly sown into a fully-prepared paddock rather than prepared strips owing to the abundance of cultivated paddocks used for growing annual crops or forages and the lower costs compared with establishing into an existing grass paddock. Planting times are generally earlier (spring to early summer) than in central Queensland (mid-summer to early autumn) to maximize the size, therefore the robustness, of the plant before the onset of winter. Many paddocks sown to leucaena in southern Queensland are deficient in critical soil nutrients owing to the long history of cropping and so have a high fertilizer requirement.

Leucaena has not been adopted in northern New South Wales and is currently not recommended owing to its weed potential (Boschma et al. 2018). Given the large areas of cropping land and the presence of a farming culture, many approaches used to establish leucaena in central and southern Queensland should be applicable for New South Wales if/when leucaena is seen as a viable forage option in that State.

North Queensland

Of the estimated 1,500 ha of leucaena recently (since 2015) planted, only about 900 ha has established successfully, emphasizing some region-specific risks associated with sowing leucaena in more northern environments and the continued need to improve producer skills and refine establishment practices. The adoption of the new cultivar Redlands is being treated with caution until its commercial productivity in northern environments is confirmed in current grazing experiments. This follows previous research in north Queensland where Redlands was found to be less palatable to cattle than other commercial but psyllid-susceptible varieties (Mark Keating pers. com. 2018).

In north Queensland the larger areas of cleared soils for conventional leucaena establishment are confined to deep soils of the Atherton Tablelands and alluvial soils along the wet coast (coastal soils with >900 mm mean annual rainfall). Smaller areas of cleared basalt and alluvial soils in the seasonally dry tropics are also well suited to leucaena. The challenges associated with establishment of leucaena in the seasonally dry tropics of north Queensland include the difficulties associated with rocky basalt soils and generally more extreme conditions

for sowing and establishment than in southern areas. These include high evaporation rates, lighter soils with lower moisture holding capacity, higher temperatures and the greater intensity of rainfall during the wet season.

Recommended practices for planting and establishing leucaena in central Queensland are generally transferable to north Queensland situations for conventional leucaena establishment into cleared paddocks, but this is not the case for large areas of timbered country, which are otherwise suited to leucaena. Leucaena establishment in the northern rocky basalt province was pioneered during the 1990s by Greg Brown, a beef producer on Meadowbank Station, on timbered country that was cleared by stem-injection. Planting leucaena into this situation doubled beef productivity compared with that obtained from native grass-only pastures (Buck et al. 2019). There are 2.3 million hectares of such high-phosphorus basaltic woodlands in north Queensland (Isbell et al. 1976), representing a significant opportunity for leucaena development. However, while current vegetation management legislation now prevents the removal of trees from this landscape, leucaena can be successfully established in these woodland environments with appropriate cultural practices (Mark Keating pers. comm. 2018). Following deep ripping of strips between the existing trees during the dry season, leucaena has been sown successfully using a custom-built, heavy-duty planter (single row). Other problems associated with these rocky basalt soils are: cultivation for weed control is impractical except at planting time; weed control is virtually totally dependent on the availability and effectiveness of pre- and post-emergent herbicides; and ongoing fertilizer applications appear essential to overcome inherent sulphur deficiencies in these soils. Once leucaena is established on these timbered and rocky landscapes, ground-based applications of sulphur are impractical (except immediately after heavy pruning) so aerial application is the only practical method. While leucaena can be successfully established, the long-term productivity (and profitability) of leucaena in these lightly-timbered environments, where trees compete for nutrients, moisture and light, is unknown.

Light-textured soils in north Queensland also pose particular challenges for establishing leucaena. Sowing depth and moisture availability are critical with greater success observed when seed is placed at depths no greater than 25 mm with ample soil moisture. Pre-emergent weed control with the current suite of herbicides is limited by the prevalence of sown legumes such as *Stylosanthes* spp. and *Chamaecrista* spp. in the northern seasonally dry tropics. Finally, full-paddock cultivation for leucaena establishment is generally avoided owing to the difficulty of cultivation in rocky soils (where cleared), erosion risk on lighter soils and cost and difficulty in re-establishing inter-row pastures in northern environments.

Northern Territory and Western Australia

Very small areas of leucaena have been established in other areas of northern Australia and only the Northern Territory currently contains any area of significance (Buck et al. 2019). The Katherine and Douglas Daly regions in the Northern Territory, and the Ord irrigation area in the Kimberly region of Western Australia are reported to contain suitable soils for leucaena (Peter Shotton and Clinton Revell pers. comm. 2018). Establishment methods are similar to those used in Queensland and include: removal of the existing pasture/crop and fallowing during the wet season to conserve soil moisture; seedbed preparation and weed control by cultivation; post-planting weed control with residual herbicide; accurate seed placement with suitable planter; and withholding of grazing for up to 12 months until fully established.

Conclusions

Leucaena is a highly productive perennial tree legume but the area currently established in northern Australia is very small compared with the potential. Suitable methods for establishing this valuable browse species in fertile cleared areas have been developed. Key practices include a friable seedbed, stored soil moisture prior to sowing, effective weed control, soil fertility management, timely sowing with high-quality seed and withholding grazing until fully established. Ongoing studies will develop suitable techniques for expansion into timbered situations and less-fertile soils in northern Australia with greater confidence. However there are still significant aspects which require elucidation to take full advantage of what leucaena has to offer, e.g. optimal fertilizer requirements, long-term productivity in competition with trees, production of sterile varieties to combat weediness risks and optimal inter-row spacing for differing situations.

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(Note of the editors: All hyperlinks were verified 6 May 2019.)

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Environmental adaptation of leucaena in Western Australia – challenges and opportunities

Adaptación ambiental de leucaena en el estado de Western Australia – retos y oportunidades

CLINTON REVELL[1], GEOFF MOORE[1], DANIEL REAL[1] AND SAM CROUCH[2]

[1]*Department of Primary Industries and Regional Development, South Perth, WA, Australia.* dpird.wa.gov.au
[2]*Department of Primary Industries and Regional Development, Broome, WA, Australia.* dpird.wa.gov.au

Abstract

There is considerable interest from Western Australian (WA) pastoralists on the potential role of leucaena (*Leucaena leucocephala*) in northern WA, where the potential area for dryland production of species of the genus *Leucaena* is high. Although it is highly regarded for animal production in other countries and in Queensland, leucaena is a contentious species since its status as an environmental weed currently precludes it from use on pastoral leases in the Kimberley and Pilbara regions of WA. Development of sterile/seedless forms would overcome risks of spread of the species as a weed. The key environmental constraints to growth of leucaena are likely to be the length of the dry season and low fertility of most soils other than the grey/black cracking clays (vertosols). Psyllid resistance and cool temperature tolerance are likely to be of secondary importance. Opportunities for irrigated production are also emerging and may allow leucaena species to be used in environments previously considered well outside their home-range. It is desirable now to re-examine the diversity of the wider leucaena genus for adaptation to WA conditions generally and for the purpose of selecting elite parent material for use in a sterile/seedless leucaena breeding program. These perennial species that can be under production for 30 to 40 years need to be evaluated in the target environments for at least 3–5 years to fully understand their potential as adult plants.

Keywords: Breeding, climate, shrub legumes, soil, stress, tropics.

Resumen

En el estado de Western Australia (WA), existe un gran interés por parte de los ganaderos en el uso de leucaena (*Leucaena leucocephala*) debido al considerable área potencial para la producción de especies del género Leucaena en tierras de secano. Aunque es muy apreciada para la producción animal en otros países y en el estado de Queensland, leucaena es una especie muy discutida ya que su condición de maleza ambiental excluye actualmente su uso en tierras oficiales arrendadas para explotación pastoril en las regiones de Kimberley y Pilbara en WA. El desarrollo de formas estériles/sin semillas superaría los riesgos de diseminación de la especie como maleza. Las restricciones ambientales clave para el crecimiento de leucaena probablemente sean la duración de la estación seca y la baja fertilidad de la mayoría de los suelos que no sean de arcillas expansivas (vertisoles). La resistencia a los psílidos (insectos de la familia Psyllidae) y la tolerancia de temperaturas bajas son probablemente de importancia secundaria. Existen oportunidades para la producción bajo riego la cual permitiría que las especies de leucaena sean utilizadas en ambientes que antes se consideraban fuera de su área de adaptación. Se considera deseable volver a examinar la diversidad del género *Leucaena* respecto a su adaptación a las condiciones de WA en general y con el fin de seleccionar líneas elite para su uso en proyectos de fitomejoramiento para desarrollar variedades de leucaena estériles/sin semillas. Debido a que estas especies perennes pueden ser productivas durante 30–40 años, se considera que deben evaluarse en diferentes condiciones ambientales durante al menos 3–5 años para comprender completamente su potencial como plantas adultas.

Palabras clave: Clima, estrés, fitomejoramiento, leguminosas arbustivas, suelos, trópico.

Correspondence: C. Revell, Locked Bag 4, Bentley Delivery Centre, Perth, WA 6983, Australia.
Email: clinton.revell@dpird.wa.gov.au

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Introduction

Leucaena is a genus of 24 recognized leguminous hardwood species in the mimosoid sub-family, native to tropical regions of Central America (Govindarajulu et al. 2011). The genus is recognized internationally as a source of multipurpose trees, of great significance for timber, forage and green manure (Brewbaker 2016). In Australia, particularly Queensland, considerable public and private investment has been directed towards adoption of leucaena forage systems for the beef industry (Beutel et al. 2018). The most widespread species *L. leucocephala* (Lam.) de Wit has been the focus of breeding efforts over 50 years, particularly centered on the subspecies *glabrata* (Rose) Zárate. All commercial cultivars in Australia have been derived from this species (cvv. Peru, Cunningham, Tarramba and Wondergraze) or interspecific crosses between this species and *L. pallida* (cv. Redlands). These cultivars are well-regarded for their high feed quality (e.g. Garcia et al. 1996; Dalzell et al. 2006) and ability to increase beef production (e.g. Davidson 1987; Pratchett and Triglone 1989; Shelton and Dalzell 2007; Bowen et al. 2016).

There is renewed interest from Western Australian (WA) pastoralists on the potential role of leucaena in northern WA, given the observed benefits to cattle producers in Queensland. However, *L. leucocephala* is a contentious species because it can become a serious environmental weed (e.g. PIER 2002; Walton 2003).

Adaptation of *Leucaena leucocephala*

The agronomic requirements for successful production of *L. leucocephala* have been widely documented (e.g. Dalzell et al. 2006; Brewbaker 2016). It is well adapted to hot and humid climates with mean annual rainfall between 650 and 1,500 mm. Pratchett and Triglone (1989) suggest that it typically requires about 750 mm of rainfall to establish, but once established it can survive on less rain and will persist through drought by shedding its leaves. A recent Australian study (Radrizzani et al. 2016) demonstrated the marked influence of amount of seasonal rainfall and age of the stand on yield of *L. leucocephala*. Yields over a 6–7 month growing season and rainfall-use efficiency were highest in 8-year-old stands [2,128 kg total dry matter (DM)/ha or 4.0 kg DM/ha/mm] and lowest in 38-year-old stands (978 kg total DM/ha or 1.9 kg DM/ha/mm). The reduced yield (a function of fewer stems per plant) and vigor over time were associated with declining soil fertility.

Maximum yields of *L. leucocephala* require daytime temperatures above 30 ºC; if night temperatures drop below 17 ºC, yields are severely reduced (Pratchett and Triglone 1989). Mullen et al. (2003a) suggest subtropical environments with very high maximum temperatures tend to have lower productivity than humid-tropical locations with moderate maximum temperatures. While leucaena species are generally limited ecologically to frost-free ecosystems (Brewbaker 2016), *L. leucocephala* can survive frost, even though leaf and stems may be killed to ground level, recovering in spring with warmer temperatures. Annual biomass production is greatly reduced in these circumstances and the search for enhanced low temperature and frost tolerance remains an important breeding objective.

Leucaena leucocephala is favored by deep fertile soils (Cooksley and Goward 1988) that store adequate soil moisture for the extensive root system to exploit (Poole 2003 cited in Radrizzani et al. 2010). Like most tropical trees, it flourishes in soils that are at least seasonally well-drained and is poorly tolerant of waterlogging and flooding (Brewbaker 2016). Leucaena species have evolved and are largely confined to regions of neutral or alkaline soils. In his review, Brewbaker (2016) describes limiting factors that include acidity per se, associated toxicity of aluminum and manganese, and deficiencies of nutrients including calcium, magnesium and phosphorus. Growth is severely reduced at pH (H_2O) levels below 5.2 and 40–50% Al saturation (Mullen et al. 2003a).

Leucaena leucocephala has high P and S requirements (Ruaysoongnern et al. 1989; Radrizzani et al. 2010) with deficiencies reducing levels of nitrogen fixation, particularly when occurring together. Radrizzani et al. (2010) found that productivity, N_2 fixation and N status of a 31-year-old stand increased with application of P and S fertilizers. Radrizzani et al. (2011; 2016) concluded that leaf analysis could be used with confidence to assess nutrient status, provided the youngest fully expanded leaf was sampled from actively growing plants in the vegetative phase of development that had received rainfall/irrigation in the preceding 28 days and the leaves were <21 days of age. Critical nutrient concentrations derived from this work are in the range of: N (3.5–4.0% DM), P (0.18–0.20% DM), K (0.8–1.0% DM), S (0.20–0.24% DM), Ca (0.25–0.35% DM), Mg (0.16–0.20% DM), Cu (2 ppm) and Zn (8–12 ppm).

Productivity of *L. leucocephala* is strongly influenced by the occurrence of the leucaena psyllid (*Heteropsylla cubana*) with yields reduced by as much as 65% by severe infestations (Mullen and Shelton 2003). Psyllids are small (3 mm) sap-sucking plant lice, which feed from the phloem of developing shoots and young foliage, so that damage is concentrated in these regions (Hughes 1998). A female can lay up to 400 eggs that mature rapidly through 5 nymphal stages in a cycle of about 2 weeks.

Under ideal conditions (warm, calm, moist) for the pest, the population increase can be logarithmic. Populations are lower in cool dry seasons, and heavy rains or sustained drought reduce nymph populations (Brewbaker 2016). Psyllids are not regarded as a serious pest in subhumid areas with 600–800 mm annual rainfall (Shelton and Jones 1995).

Adaptation of the *Leucaena* genus

There may be opportunities to overcome constraints to *L. leucocephala* production through exploiting the wider diversity in the *Leucaena* genus (Shelton and Jones 1995). Twenty-four species have been described ranging from 3 to 25 m in height at maturity and originating from elevations of 100–1,800 m (Hughes 1998; Govindarajulu et al. 2011; Brewbaker 2016). Mullen et al. (2003b) reported a genotype (116 accessions) × environment study with sites in Brisbane (subtropical Australia) and Los Baños (humid-tropical Philippines) that highlighted substantial variation within and between species for DM yield. Main effects were moderated by the influence of seasonal temperatures, rainfall and psyllid pressure as previously discussed. The evaluation of germplasm in the target environment is critical as the authors note that the Los Baños environment presented none of the constraints that commonly limit growth of *L. leucocephala*, such as low temperatures, low rainfall (drought), acid soils and high psyllid pressure. *Leucaena leucocephala* and interspecific hybrids (*L. leucocephala* × *L. diversifolia* and *L. leucocephala* × *L. pallida*) were particularly productive. There was generally a strong relationship between total DM production and edible dry matter (leaf + stem <6 mm in diameter). Other high-yielding accessions at the Brisbane site included representatives of *L. pallida*, *L. diversifolia*, *L. trichandra*, *L. lanceolata* and *L. macrophylla*. Universally low-yielding accessions originated from *L. retusa*, *L. confertiflora* and *L. greggii*. Although *L. collinsii* ssp. *collinsii* established well, it showed only moderate productivity subsequently, but nevertheless was considered a potential species for creating interspecific hybrids (valued for its psyllid tolerance and low levels of condensed tannins and mimosine). A subset of 25 accessions were grown across a range of other tropical and subtropical environments including Kununurra, Western Australia. Top-ranking accessions at Kununurra were similar to those which ranked highly at Los Baños. These assessments were made over a 2–2.5 year period (6–14 month establishment period followed by multiple harvests over the following 12 months). Although establishment growth appears to be positively correlated with post-establishment growth (Mullen et al. 2003a), it is not known whether the relative performance of species (particularly focussing on edible dry matter) would change over the long term. Furthermore, while a strain of *Rhizobium* (CB3060) known for its effectiveness across a range of leucaena species was used in these studies, it is not optimal for all species (Mullen et al. 1998) and poor nodulation and N_2 fixation could also limit the performance of some species. It is imperative that rhizobial effectiveness is accounted for in future species development.

The ability to tolerate regular cutting is an important characteristic for persistence – some accessions of *L. pallida*, *L. trichandra* and *L. collinsii* did not persist with a cutting regime of 3-4 harvests/yr after a 10 month establishment period in the work of Mullen et al. (2003b). These authors also highlighted the issue of the trade-off between the arboreal nature of the 'giant' leucaena (*L. leucocephala* ssp. *glabrata*) and the need for increased management to keep plants at a grazing height. Highly forked (multi-stemmed) forms, particularly after cutting, are desirable, and variability exists within and between species for this trait (Hughes 1998), though it has not been widely researched. *Leucaena confertiflora*, *L. cuspidata*, *L. trichandra*, *L. trichodes* and the 'shrubby' *L. leucocephala* ssp. *leucocephala* are regarded as less arboreal.

In the context of cold tolerance as a desirable trait, cool tolerance needs to be distinguished from frost tolerance (and the frequency of frosts). True frost tolerance exists in *L. greggii* and *L. retusa* (Hughes 1998; Brewbaker 2016). Interestingly, the highland species such as *L. diversifolia*, *L. pallida* and *L. trichandra* show cool tolerance but little frost tolerance and are inferior in frost tolerance to *L. leucocephala* ssp. *glabrata*. There appears to be little variability among species for tolerance to soil acidity though *L. diversifolia* and *L. pallida* appear to be more tolerant of acidity than other species (Brewbaker 2016). Species with the potential to cope with an extended dry season (7–8 months) include *L. retusa* (Brewbaker 2016) and *L. collinsii* ssp. *collinsii* and ssp. *zacapana* and some varieties of *L. pallida* (Hughes 1998).

An analysis of psyllid resistance has been reported by Mullen et al. (2003c) utilizing the genotype × environment study in Australia and the Philippines previously described. There was considerable variation in psyllid resistance both between and within some species, notably *L. trichandra*, *L. diversifolia*, *L. collinsii* and *L. pallida*. *Leucaena collinsii* ssp. *collinsii*, *L. confertiflora*, *L. esculenta*, *L. pueblana*, *L. retusa*, *L. greggii* and *L. matudae* were highly resistant in both countries, while *L. leucocephala*, *L. lempirana*, *L. involucrata* and *L. multicapitula* were highly susceptible in both countries. There was little variation for psyllid resistance within *L. leucocephala*.

Any development of alternative leucaena species needs to take account of both nutritive value and palatability for animal growth together with animal health (Hughes 1998; Stewart and Dunsdon 1998). The 24 species of leucaena can be divided on the basis of average concentrations of the mild toxin (non-protein amino acid) mimosine into a low group (~2% DM) and a high group (~4% DM) (Brewbaker 2016). The low group includes *L. collinsii, L. diversifolia, L. esculenta, L. greggii, L. pallida* and *L. pulverulenta*. Variation for in vitro dry matter digestibility (IVDMD) occurs between and within species, among tissues sampled, and among samples in different seasons and growing conditions (Stewart and Dunsdon 1998; Brewbaker 2016). Species with desirable IVDMD (>70%) include *L. collinsii, L. leucocephala, L. macrophylla, L. salvadorensis, L. trichodes, L. diversifolia, L. multicapitula, L. retusa* and *L. shannonii*. Stewart and Dunsdon (1998) developed forage quality indices for a range of leucaena taxa (each represented by only a single accession) based on a combination of laboratory analysis (crude protein and digestibility), biomass and palatability using pen-fed sheep. While this was a relatively limited study, high-scoring species included *L. leucocephala* ssp. *glabrata, L. collinsii* ssp. *zacapana, L. shannonii* ssp. *shannonii* and *L. diversifolia*, with *L. leucocephala* ssp. *glabrata* and *L. diversifolia* notable for their superior animal preference. Condensed and total tannins also vary widely between species (lowest in *L. collinsii*), and high levels appear to reduce dry matter digestibility in the rumen (Stewart and Dunsdon 1998). Clearly, more research to understand and exploit the variability in nutritive value within and between the entire range of leucaena species is required.

A short history of leucaena in Western Australia

Commercial sowings of leucaena in WA have been limited. The notable exception was on the black cracking clay soils of the Ord River Irrigation Area (ORIA) in the 1980s to early 1990s, where *L. leucocephala*, predominantly cv. Cunningham (Bolam et al. 1998), was successfully grown (~1,400 ha). High beef production of 1,400–1,500 kg liveweight gain/ha/yr was measured on leucaena-pangola grass (*Digitaria eriantha*) pastures in the ORIA under flood irrigation (Davidson 1987; Pratchett and Triglone 1989). Davidson (1987) reported a liveweight gain per head of 237 kg over 12 months and Bolam et al. (1998) reported individual growth rates of up to 690 g/hd/d under irrigation in the dry season. A series of grazing trials on the Frank Wise Institute at Kununurra evaluated different stocking rates, different row configurations (including close row spacing as cattle were eating predominantly *L. leucocephala*) and meat quality (Pratchett and Triglone 1989; Pratchett et al. 1992). Beef production appeared to increase over time providing stands were not over-grazed in the establishment years (particularly for close row spacings).

The commercial plantings of *L. leucocephala* in the ORIA have subsequently been replaced by forestry (sandalwood) and horticultural crops due to more favorable economics rather than through problems associated with agronomy or productivity (Brann 2008). However, as a result of management practices, overland water flow in the wet season and/or over-watering with flood irrigation, seeds of *L. leucocephala* have entered the waterways of the Ord River and it is now a weed of riparian zones (Walton 2003).

We are not aware of any other commercial plantings of leucaena species in the Kimberley, or elsewhere in WA. *Leucaena leucocephala* has been planted for shade around homesteads and roadhouses in the Kimberley, Pilbara and Gascoyne and is present in highly disturbed environments like town sites (Walton 2003). The Western Australian Herbarium (1998) describes *L. leucocephala* as an alien species, which is present in the central and northern Kimberley, Murchison and Pilbara. The vast majority (>98%) of grazing land in northern WA is under pastoral lease and a diversification permit from the Pastoral Lands Board is required to grow any non-indigenous plants. The approval process includes a weed risk assessment. *Leucaena leucocephala* has been assessed as a 'very high' environmental weed risk for both the Pilbara and Kimberley (Randall 2018) and is currently not approved for use on pastoral leases in these regions. This outcome aligns with widespread findings on the weed potential of *L. leucocephala*. For example, Lowe et al. (2000) include *L. leucocephala* in a list of 100 of the world's worst invasive alien species. Richardson and Rejmánek (2011) include *L. leucocephala* as 1 of only 6 trees or shrubs known to be invasive in 10 or more regions of the world (12 regions including Australia). Randall (2012) reports it as a weed of the natural environment, escaping from cultivation, and an invasive species in Australia. In contrast with Queensland where the issues with weediness are largely attributed to *L. leucocephala* ssp. *leucocephala* (Walton 2003), in WA the weed issue is with naturalized *L. leucocephala* ssp. *glabrata*. Recent observations by the authors in northern WA are of individual plants with high seed production and often with seedling recruitment.

Potential role for *Leucaena* species in WA

The potential role for leucaena-based pastures in northern WA is unclear, even if the weed risk could be reduced,

such as through the development of a sterile or seedless cultivar. There are questions about its adaptation to environments outside the ORIA and the agronomic practices and soil amelioration that would be required to increase productivity. All species of leucaena are permitted into WA, but *L. leucocephala* and *L. lanceolata* have been assessed as very high weed risks in the Kimberley and Pilbara regions and their use is problematic.

The environment in northern WA

The rangelands of northern Western Australia cover a broad range of climatic zones and soil types, predominantly spanning latitudes 16–24° S. The average annual rainfall (AAR) varies from >1,100 mm in the north Kimberley to <300 mm in the southern Pilbara (Figure 1).

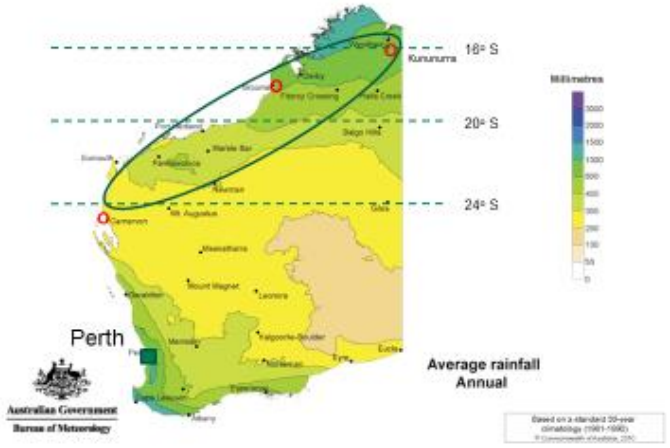

Figure 1. Target environment for leucaena in WA (circled). Source: Bureau of Meteorology, Australian Government.

Based on a modified Köppen climate classification system the Kimberley is predominantly categorized as 'Tropical–savanna', while the southern section of the Kimberley and the Pilbara are classified as 'Grassland–hot with winter drought' (BoM 2001; 2014). The regions have a distinct wet season from October–December to February–April and a dry season with little or no precipitation from March–April through to November–December.

Annual rainfall is increasing in the Kimberley. CSIRO (2009) determined that the recent climate period of 1996–2007 in the central Kimberley was 31% wetter than the historical period of 1930–2007. Wet season rainfall is highly variable, influenced in part by the strength of monsoon systems and the occurrence of sporadic cyclones. Wet season temperatures are also high, with average daily maximum temperatures typically ranging between 33 and 39 °C (BoM 2016). Dry season temperatures are milder with average daily maximum temperatures between 24 and 33 °C and average daily minimum temperatures between 12 and 18 °C. Inland regions can experience night temperatures as low as 5 °C.

The dominant soil types include grey/black cracking clays (vertosols) along the major river systems (flood plains), areas of red earths (red kandosols) in the north Kimberley and large areas of red-brown sandy soils (red-orthic tenosols), especially in the west Kimberley and Pilbara. A summary of the main soil groups by rainfall classes (>1000 mm, 800–1,000 mm and 600–800 mm AAR) for the Kimberley Region, the most likely dryland target, is provided in Table 1. The soil groups are broad categories and there is substantial variation within each group, which influences their agronomic potential (Schoknecht and Pathan 2012). For example, in the La Grange area in the west Kimberley Smolinski et al. (2016) identified 5 variations within 'Cockatoo sands' which are red-brown sands (colloquially known as 'Pindan' sands). They report the topsoil in these soils as relatively uniform (i.e. red-brown sands to loamy sands), so differences relate mainly to changes in texture down the soil profile. The subsoil texture varies considerably, which affects the plant-available water holding capacity (e.g. PAWC 50–108 mm in the top metre of soil; Smolinski et al. 2016).

Most of the soils in northern WA are inherently infertile with very low phosphorus, potassium and soil organic carbon levels (Table 2). Pindan sands have a very low cation exchange capacity (CEC) of 2 meq/100g in the topsoil, while the soil phosphorus retention index (PRI) is also low (typically <7) and positively correlated with soil clay content.

Table 1. A summary of the areas of the main soil groups (,000 ha) by average annual rainfall (AAR) for the Kimberley Region, Western Australia.

AAR (mm)	Loamy earths	Loamy duplexes	Sandy duplexes	Deep sands	Cracking clays	Non-cracking clays	Sandy earths	Total
>1,000	257	203	158	515	59	5	104	1,301
800–1,000	396	34	103	753	557	20	352	2,215
600–800	399	67	145	1,222	762	53	555	3,203

Table 2. Indicative properties of the dominant soil groups in the Kimberley Region, Western Australia.

Soil group	Clay content (0–10 cm; %)	Org. C (%)	Surface pH (1:5 water)	Phosphorus (Colwell; ppm)	Potassium (Colwell; ppm)
Loamy earths	7–13	0.2–0.9	6.0–6.9	<2–3	20–180
Loamy duplexes	10	0.7	6.7	<2	170
Deep sands	4–12	0.15–0.5	5.8–7.0	<2	20–30
Cracking clays	40–65	0.4–0.9	6.8–8.5	2–10	100–300
Sandy earths	5–10	0.2–0.6	5.7–6.9	<2	15–50

Opportunities for northern WA

The potential area suitable for dryland sterile leucaena in northern WA is high. There are about 5.4 M ha of soils within the 600–1,000 mm rainfall zone (Table 1), of which about 40% would potentially be suitable for leucaena. However, unlike eastern Australia, the proportion of cleared/arable land for leucaena establishment is currently very small, perhaps only in the thousands of hectares (outside the ORIA). Flood plains of the larger river systems and the grasslands of old marine sediments have less woody vegetation, but can be inundated for long periods in years when wet season rainfall is above average. Site selection in these environments would be critical. In addition, freehold land represents less than 2% of the area and General and Special lease tenure represents less than 1%, with the remainder under national parks and pastoral lease (not all pastoral lease is actively managed for cattle production). Any intent to establish a sterile leucaena on pastoral lease would still require regulatory approval through both diversification and clearing permits.

While land (often comprising Pindan sand) is now being developed with irrigation (Ash et al. 2017; MacLeod et al. 2018), the potential area for irrigated leucaena will be limited by the availability of water as well as any soil constraints and competition from other land uses (e.g. horticulture, broad-acre crops, forestry and fodder species). The maximum area for irrigated sterile leucaena production is likely to be <10,000 ha.

In WA the key environmental constraints are likely to be the length of the dry season and low fertility of most soils other than the grey/black cracking clays (vertosols). We have also observed significant plant losses from termites (including *Mastotermes darwiniensis*) in field trials and these could pose a further constraint on some soils. Management of other grazing herbivores such as wallabies will also be required. Psyllid resistance and cool temperature tolerance are likely to be of secondary importance. While existing commercial cultivars of *L. leucocephala* are currently not approved for use on pastoral lease, it is desirable now to re-examine the diversity of the wider leucaena genus for adaptation to WA conditions generally and for the purpose of selecting elite parent material for use in a sterile/seedless leucaena breeding program. These perennial species that can be under production for 30–40 years need to be evaluated in the target environments for at least 3–5 years to fully understand their potential as adult plants.

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(Note of the editors: All hyperlinks were verified 17 April 2019.)

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ILC2018 Keynote paper*

Leucaena shows potential in Northern Inland New South Wales, Australia

Leucaena muestra potencial en el norte del interior de New South Wales, Australia

CAROL HARRIS[1], SUZANNE BOSCHMA[2], MARK BRENNAN[2], LAUREN BORG[3], STEVEN HARDEN[2] AND BRIAN CULLIS[3]

[1]*NSW Department of Primary Industries, Glen Innes, NSW, Australia.* dpi.nsw.gov.au
[2]*NSW Department of Primary Industries, Calala, NSW, Australia.* dpi.nsw.gov.au
[3]*Centre for Bioinformatics and Biometrics, National Institute for Applied Statistics Research Australia, University of Wollongong, Wollongong, NSW, Australia.* niasra.uow.edu.au

Abstract

A study was conducted during 2013–2017 to evaluate the potential of 5 cultivars/experimental lines of leucaena (*Leucaena leucocephala*) at 2 sites in Northern Inland NSW. In this frost-prone, summer-dominant rainfall region, all cultivars/lines established well and survival was >70% at Bingara and >95% at Manilla. Cultivars Wondergraze and Cunningham were the most productive, producing up to approximately 2.4 t DM/ha and 1.9 t DM/ha per growing season at Bingara and Manilla, respectively. Tropical grass establishment in the alleys was poor with plant productivity inversely related to leucaena productivity. Although this study has confirmed the persistence and productive potential of leucaena, the challenges around tropical grass establishment and persistence as well as the weed potential of leucaena in this region need to be addressed before broad-scale use could be recommended in Northern Inland NSW.

Keywords: *Digitaria eriantha*, persistence, tree legumes, variance components analysis.

Resumen

Se realizó un estudio para evaluar el potencial de cinco cultivares/líneas experimentales de leucaena (*Leucaena leucocephala*) en dos sitios en la región norte del interior de NSW durante 2013–2017. En esta región, que se caracteriza por lluvias en verano y ser propensa a heladas, todos los cultivares/líneas se establecieron bien y su supervivencia fue >70% en Bingara y >95% en Manilla. Los cultivares Wondergraze y Cunningham fueron los más productivos, alcanzando hasta 2.4 t MS/ha y 1.9 t MS/ha por época de crecimiento en Bingara y Manilla, respectivamente. El establecimiento de la gramínea tropical asociada (*Digitaria eriantha*) fue deficiente y su producción estuvo inversamente relacionada con la de la leucaena. Aunque este estudio ha confirmado el potencial de persistencia y productividad de la leucaena, antes de poder recomendar su uso a mayor escala en el interior del norte de NSW es necesario abordar los desafíos relacionados con el establecimiento y la persistencia de las gramíneas tropicales asociadas, así como el potencial de la leucaena de volverse una maleza invasiva en esta región.

Palabras clave: Análisis de la varianza de componentes, *Digitaria eriantha*, leguminosas arbóreas, persistencia.

Introduction

Northern Inland New South Wales (NSW) is a subhumid summer rainfall zone (Tweedie and Robinson 1963) with approximately 60% of annual rainfall falling between October and March, commonly in high-intensity thunderstorms. Pasture growth in the region is limited by low temperatures in winter and high temperatures and soil

Correspondence: C.A. Harris, Glen Innes Agricultural Research and Advisory Station, 444 Strathbogie Road, Glen Innes, NSW 2370, Australia. E-mail address: carol.harris@dpi.nsw.gov.au

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moisture stress during summer (Harris and Culvenor 2004). The average period of frost occurrence is 145 days with approximately 50 frosts received per year (Hobbs and Jackson 1977), while summer rainfall tends to be relatively ineffective as high summer temperatures lead to high evapotranspiration (Murphy et al. 2004).

In the Northern Inland mixed farming zone of NSW sown grass pastures were traditionally based on temperate species (e.g. Archer 1989; Lodge and Orchard 2000; Harris and Culvenor 2004); however, they have now largely been replaced by tropical grasses (Harris et al. 2014). To maintain the productivity of tropical pastures, soil nutrients, in particular nitrogen, are required (Boschma et al. 2014), which can be applied as inorganic sources and by addition of a companion legume. Research has been conducted in Northern Inland NSW to expand the range of legume options available as a companion to the tropical perennial grass-based pastures. Leucaena was included in this research based on its productivity and persistence in an experiment established at Tamworth in January 2009 (S.P. Boschma unpublished data).

As part of these studies an experiment was conducted at 2 sites in the North West Slopes region of NSW to evaluate 4 cultivars and an experimental line of leucaena in a mix with digit grass (*Digitaria eriantha* cv. Premier). The agronomic traits used to evaluate suitability were establishment, persistence and herbage production of both leucaena and tropical grass over 4 years (2013–2017).

Methodology

Sites

The experimental sites were located near Bingara (29º42'39" S, 150º27'07" E; 297 masl) and Manilla (30º42'11" S, 150º30'10" E; 412 masl) in Northern Inland NSW. Some site characteristics are shown in Table 1. For 2 years prior to the commencement of the experiments both sites were sown to winter oats (*Avena sativa*) with a summer fallow. In the spring prior to establishment of the experiments, weeds were sprayed with glyphosate (450 g/L a.i. at 1.5 L/ha).

Table 1. Long-term average annual rainfall (AAR, mm), soil type and some soil chemical properties [pH (pH_{Ca}, $CaCl_2$), phosphorus (P, Colwell, mg/kg) and sulphur (S, KCl_{40}, mg/kg)] for the experimental sites in Northern Inland NSW.

Site	AAR (mm)	Soil type	Soil chemical properties (0–10 cm)		
			pH_{Ca}	P	S
Bingara	742	Brown Chromosol	5.0	50	3.2
Manilla	576	Brown Chromosol	6.1	36	4.2

Species, sowing and experimental designs

Four commercial cultivars of leucaena (Wondergraze, Cunningham, Tarramba and Peru) and an experimental line developed by the University of Queensland (Expt. Line) were sourced from seed companies and the University of Queensland, respectively. Seedlings of each line were established in glasshouses in December 2012 and transplanted as 6–8-week-old seedlings into the field at Bingara and Manilla in January 2013 and watered for up to 8 days after transplanting.

The experiments were designed as randomized complete blocks with 3 replicates. Each plot consisted of 16 plants of a cultivar/line of leucaena transplanted 0.5 m apart in twin rows (1 m apart). Each row and plot was 4 m in length and each replicate was 20 m long (5 cultivars/line × 4 m each) with an additional 1 m row of leucaena (i.e. 2 plants) at each end of both twin rows as a buffer. The alley between individual replicates was 6 m.

Digit grass cv. Premier was sown in the 6 m alleys between leucaena twin rows at 1 kg/ha (viable seed) at Bingara and Manilla in December and November 2013, respectively, but failed to establish at both sites and was resown in November 2014. The grass again failed to establish at Bingara and the experiment continued at this site without a sown grass in the alleys.

Site management

At the Bingara site, grass weeds along the twin leucaena rows were controlled with haloxyfop (520 g/L a.i. at 100 mL/ha) in August 2014 and 2015. The alley between leucaena rows was maintained in weed-free fallow with 3 applications of glyphosate (1.5 L/ha) during the period April–November 2013. After 2015, grass and broad-leaf weeds were controlled in alleys with glyphosate (1.5 L/ha) and 2,4-D ester (680 g/L a.i. at 1.3 L/ha) on 3 occasions.

At the Manilla site, grass weeds along the twin leucaena rows were controlled with fluazifop-P (128 g/L a.i. at 0.5 L/ha) in February 2013. Imazethapyr (700 g/kg a.i. at 70–100 g a.i./ha) was applied as granules in July and December 2013, and July and October 2015 to provide residual weed control. The alley between leucaena rows was maintained in weed-free fallow with 5 applications of glyphosate (1.5 L/ha) during the period April–November 2013. Broad-leaf weeds in digit grass were controlled with 2,4-D ester (720 g/L a.i. at 1.7 L/ha) in June 2015.

At both sites single superphosphate (8.8% P, 11% S) was applied at 200 kg/ha in spring-early summer each year from 2013. In September each year, as leucaena plants were recommencing growth, the dead frosted stems were cut to a height of 0.3 m and the woody material removed from the

experiment. Leucaena pods were removed and destroyed to eliminate the risk of recruitment throughout the experiment.

Data collection

Rainfall data at both sites were recorded manually. Long-term average monthly and annual rainfall data for both sites were extracted from Bureau of Meteorology sites.

Leucaena establishment and persistence. Plant numbers were recorded 2–3 months after transplanting in the paddock to determine establishment success. Persistence of individual leucaena plants was assessed in spring and autumn each year by recording their presence and health.

Leucaena herbage production. Leucaena herbage mass was assessed from late spring/early summer to autumn each growing season, whenever the tallest leucaena plants reached approximately 1.8 m in height. At each assessment the number of stems was recorded for 8 plants, i.e. the middle 4 plants in each row except when the plants were not representative of those in the plot. A representative stem on each assessed plant was selected, cut at the point where the stem diameter was about 10 mm and bagged. All leaves from the remainder of this stem were also removed to the base of the plant and placed in the same bag. The harvested stem plus leaf material represented the edible portion of the plant and was dried in a dehydrator for 48 h at 80 ºC, then weighed to calculate herbage dry weight (kg DM/ha). After each assessment all leucaena plants were cut back to a height of 0.5 m and material removed from the plots. Herbage mass was assessed 1, 3, 3 and 4 times (total 11 times) at Bingara and 1, 2, 3 and 2 times (total 8 times) at Manilla in Years 1, 2, 3 and 4, respectively.

Grass establishment, production and persistence. Counts of seedling density (seedlings/m^2) of digit grass were taken 4–6 weeks after sowing in 6 quadrats (0.1 × 0.5 m) in each plot.

At Manilla the grass sown in the alleys was assessed at the same time as the leucaena from November 2015 using visual assessment (i.e. a total of 5 times). For each plot, 4 assessments of total herbage mass were made visually (continuous 0–5 scale, where 0 = nil and 5 = highest herbage mass) and the percentage of digit grass (dry weight herbage mass) assessed. Fifteen calibration quadrats (0.4 × 0.4 m), representing the range of herbage mass at a site were also scored, harvested to 10 mm above ground level and sorted into digit grass and other species. The samples were dried at 80 ºC for 48 h and weighed. Herbage mass scores and percentage estimates were regressed (linear or quadratic R^2>0.80) against actual herbage mass (kg DM/ha) and percentage of digit grass to determine herbage mass of the sown grass. After each assessment the plots were mown with a rotary mower and the herbage removed from the plots.

In spring and autumn each year commencing spring 2015, plant frequency of the digit grass was assessed. The proportion (%) of cells (each 0.1 × 0.1 m) containing a live plant was used to estimate frequency of occurrence (plant frequency, Brown 1954) in 2 permanent quadrats (1.0 × 0.5 m, i.e. 50 cells/quadrat) located in the alley on either side of the leucaena twin rows. Estimates were taken 0–10 days after the experimental area was defoliated.

Statistical analyses

Variance components analysis. Three traits were analyzed: leucaena herbage mass, grass herbage mass and grass frequency. Data for each combination of trait and site were analyzed individually using a variance components analysis. A linear mixed model was fitted to the data for each trait by site combination using the software ASReml (Gilmour et al. 2006) in R (R Core Team 2017).

Leucaena herbage mass, grass herbage mass and grass frequency data were cube-root transformed to more closely resemble a Gaussian distribution. Non-genetic effects associated with the experimental design of the trials were crossed with the longitudinal factor for sampling times (Brien and Demetrio 2009) and fitted as random effects. In terms of the genetic effects, the random component of the model included a main effect for legume varieties and an interaction term between sampling times and varieties and assumed a simple variance component structure for these effects. The statistical significance of genetic terms in the model was assessed using the residual maximum likelihood ratio test (REMLRT) to compare the likelihood of the full model against the model excluding the effect under examination. The resulting test statistic was then compared with the reference distribution of a mixture of chi-squared variates (Stram and Lee 1994).

Effects related to varieties were fitted as random effects and the empirical best linear unbiased predictors (BLUPs) obtained (Smith et al. 2005). The BLUPs of the overall performance for each cultivar (Smith and Cullis 2018) for each trait were calculated for each site, as well as the 90% confidence interval for these predictions. The overall performance was added to the BLUP of the overall mean for cultivars, averaged across environments. This value was then back-transformed as an approximation of the overall mean performance on the scale of the original data to provide a value for each legume treatment that was biologically meaningful. When interpreting BLUPs, the confidence intervals provided are not a formal test for comparison of treatments (i.e. significance) because treatment effects were fitted as a random effect. Instead they are a test for the true value of each treatment individually.

Leucaena persistence. There was very little change in persistence during the experiment and little variation between many of the species, so no statistical analyses were conducted.

Grass establishment. Seedling densities (seedlings/m^2) were analyzed by ANOVA with leucaena cultivar/line as the explanatory factor and replicate as a block term. Data transformation was not required.

Results

Rainfall

During the leucaena establishment period (January–April 2013), rainfall at the Bingara and Manilla sites was above average and average, respectively (Table 2). Growing season (November–April) rainfall at both sites was below average in all years, except 2014/15 at Bingara.

Leucaena establishment and persistence

Plants established successfully at both sites with 98–100% survival 2.5–3.0 months after transplanting. At the Bingara site plant numbers for all cultivars declined during the first 12 months of the experiment to 88–73%, and then remained relatively stable. At the end of the experiment cvv. Tarramba, Cunningham, Wondergraze and Peru had similar plant survival (85–81%) and Expt. Line was the least persistent at 71%. Despite the dry conditions at the Manilla site plants of cvv. Tarramba, Wondergraze and Peru persisted during the course of the experiment (i.e. maintained 100%). Small numbers of plants in the Expt. Line and cv. Cunningham died, but survival rates were 96% at the end of the experiment.

Leucaena herbage production

At Bingara cv. Wondergraze was ranked highest over the 4 years of the experiment with an average herbage mass of 2,394 kg DM/ha/assessment (back-transformed predicted mean herein referred to by units only), which was similar to cv. Cunningham (2,059 kg DM/ha/assessment). The remaining treatments had below-average productivity (BLUP<0; Table 3).

At Manilla cv. Cunningham had an average herbage mass of 1,904 kg DM/ha/assessment and was ranked highest, followed by cv. Wondergraze (1,704 kg DM/ha/assessment), with both having above average productivity (BLUP>0). The Expt. Line was ranked 5th (1,302 kg DM/ha/assessment; Table 3).

Table 2. Rainfall (mm) received during each leucaena growing season (November–April) and non-growing season (May–October) from January 2013 to April 2017, at the Bingara and Manilla sites. Long-term average (LTA) rainfall data are from Bureau of Meteorology sites Bingara (054004; 1878–1997) and Manilla (55274; 1909–2013).

Year	Bingara		Manilla	
	Growing season (Nov–Apr)	Non-growing season (May–Oct)	Growing season (Nov–Apr)	Non-growing season (May–Oct)
2013	316[1]	211	203[1]	145
2013/14	172	99	238	75
2014/15	482	252	239	200
2015/16	333	411	155	314
2016/17	362	-[2]	150	-
LTA	436	306	334	242

[1]Rainfall January–April 2013. [2]Experiment concluded April 2017.

Table 3. BLUPs (empirical best linear unbiased predictors) of the treatment effects, treatment means and their confidence intervals (CI), plus back-transformed means (scaled mean kg DM/ha/assessment) for leucaena herbage mass at the Bingara and Manilla sites.

Treatment	Bingara					Manilla				
	BLUP	Mean	CI Lower	CI Upper	Herbage mass (kg DM/ha)	BLUP	Mean	CI Lower	CI Upper	Herbage mass (kg DM/ha)
Wondergraze	0.91	13.38	12.34	14.42	2,394	0.37	11.94	11.20	12.69	1,704
Cunningham	0.25	12.72	11.68	13.76	2,059	0.82	12.40	11.65	13.14	1,904
Tarramba	-0.16	12.31	11.27	13.35	1,866	-0.06	11.51	10.77	12.26	1,526
Peru	-0.66	11.81	10.76	12.85	1,646	-0.48	11.09	10.35	11.84	1,365
Expt. Line	-0.34	12.13	11.09	13.17	1,784	-0.65	10.92	10.17	11.64	1,302

Table 4. BLUPs (empirical best linear unbiased predictors) of the treatment effects, treatment means and confidence intervals (CI), plus back-transformed means (scaled means kg DM/ha and % per assessment) for digit grass herbage mass and plant frequency at the Manilla site.

Treatment	Herbage mass					Plant frequency				
	BLUP	Mean	CI Lower	CI Upper	Back-transformed (kg DM/ha)	BLUP	Mean	CI Lower	CI Upper	Back-transformed (%)
Wondergraze	-0.28	7.28	6.69	7.88	386	-0.09	2.70	2.51	2.89	19.6
Cunningham	-0.10	7.47	6.87	8.06	416	0.01	2.79	2.60	2.98	21.8
Tarramba	0.13	7.70	7.10	8.29	456	0.04	2.83	2.63	3.02	22.6
Peru	-0.28	7.28	6.69	7.88	386	-0.08	2.71	2.52	2.90	19.8
Expt. Line	0.53	8.09	7.50	8.69	530	0.12	2.91	2.72	3.10	24.6

Grass establishment, herbage production and persistence

Establishment of digit grass at the Manilla site was poor and ranged from 4 plants/m^2 in the alley adjacent to cv. Tarramba to 0.5 plants/m^2 adjacent to cv. Wondergraze ($P>0.05$). Digit grass herbage mass varied, although the range was small; digit grass adjacent to Expt. Line was ranked highest (530 kg DM/ha/assessment), while cvv. Wondergraze and Peru had the lowest grass herbage mass (386 kg DM/ha/assessment; Table 4). Plant frequency ranking reflected herbage mass ranking with Expt. Line ranked highest (24.6%) and cv. Wondergraze ranked 5th (19.6%; Table 4).

Discussion

This study showed that leucaena can establish successfully and is persistent in Northern Inland NSW. While productivity varied with site, cvv. Wondergraze and Cunningham were consistently the most productive cultivars and Expt. Line the least. Cultivar Cunningham, bred by CSIRO and released in Australia in 1976, has good basal branching giving it a 'bushy' habit (Cook et al. 2005; Dalzell et al. 2006). Cultivar Wondergraze was bred in Hawaii, released in Australia in 2010 and has higher basal branching than cv. Tarramba.

All cultivars of leucaena established at both sites and were productive, the best cultivar producing approximately 7 and 5.3 t DM/ha per growing season at Bingara and Manilla, respectively. This confirms previous research conducted at Tamworth (S.P. Boschma unpublished data).

Leucaena is reported to have poor cold tolerance (Cooksley et al. 1988) but, while plant growth ceased over winter at both sites, plant survival was not adversely affected, demonstrating that leucaena can survive in these colder environments and be productive, despite the shorter growing season.

Establishing the tropical grass in the inter-row spaces proved challenging even though best-practice recommendations for establishing leucaena-grass pastures developed in central Queensland were followed (Dalzell et al. 2006). Dalzell et al. (2006) recommend establishing leucaena hedgerows in the first summer and then sowing grass in the following summer as leucaena has a weak seedling and is slow to establish (Lambert 2013). While this method allowed leucaena to establish well, the leucaena was highly competitive against seedling grasses in the second summer and no grass survived. Extensive cracks in the soil surface were present across the full width of the 6 m alley indicating that the leucaena had dried the soil profile. Growing season rainfall in 2013/14 was well below average at both sites. However, when grass was resown in 2014/15, when growing season rainfall was above average at Bingara, grass establishment at this site also failed. This raises the possibility of increasing the distance between the twin rows to at least 8–10 m on soils in the area similar to these sites to reduce competition from the leucaena for moisture. Failure to establish a grass in the alley may result in poor ground cover, weed invasion, increased potential for erosion and reduced livestock production (e.g. Shelton and Dalzell 2007). An alternative technique to establish a leucaena-grass pasture would be to sow both species in the same year, leaving a 2–3 m buffer on either side of each leucaena hedgerow to minimize competition between the 2 species during the first year. A similar strategy, using 1 m buffers, was found to have merit in Southern Inland Queensland (Lambert 2013).

During this study, flowers and pods were removed before pods could ripen to reduce the potential for seed spread. Leucaena has weed potential (Walton 2003a; 2003b) due to its ability to produce seed year-round (in the tropics), build a substantial seed bank, resprout after cutting or burning, tolerate drought and produce thickets (Hughes and Jones 1998). This is a biosecurity concern to

a number of state government agencies in NSW, as well as Western Australia (WA). In Queensland, The Leucaena Network (Christensen 2019) has developed a Code of Practice for managing leucaena by a combination of grazing strategy and slashing/mulching to minimize seed set; however, an effective means of overcoming weed potential is development of a seedless or sterile leucaena. A project to develop sterile lines commenced in 2017 in a collaborative exercise involving WA Department of Primary Industries and Regional Development, University of Queensland and Meat and Livestock Australia Donor Company.

Our study has confirmed the persistence and productive potential of leucaena as a summer-growing companion legume for tropical perennial grasses in Northern Inland NSW. It has, however, highlighted challenges in establishing a productive and persistent perennial tropical grass base. More research is needed to identify a suitable companion grass for this promising legume.

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ILC2018 Keynote paper*

Establishment and management of leucaena in Latin America

Establecimiento y manejo de leucaena en América Latina

NAHUEL A. PACHAS[1], ALEJANDRO RADRIZZANI[2], ENRIQUE MURGUEITIO[3], FERNANDO URIBE[3], ÁLVARO ZAPATA CADAVID[3], JULIÁN CHARÁ[3], TOMÁS E. RUIZ[4], EDUARDO ESCALANTE[5], ROGERIO M. MAURICIO[6] AND LUIS RAMÍREZ-AVILÉS[7]

[1]*School of Agriculture and Food Sciences, The University of Queensland, Brisbane, QLD, Australia.* agriculture.uq.edu.au
[2]*Instituto de Tecnología Agropecuaria (INTA), Leales, Tucumán, Argentina.* inta.gob.ar
[3]*Centro para la Investigación en Sistemas Sostenibles de Producción Agropecuaria (CIPAV), Cali, Colombia.* cipav.org.co
[4]*Instituto de Ciencia Animal (ICA), La Habana, Cuba.* ica.inf.cu
[5]*Formerly: Universidad de Los Andes, Facultad de Ciencias Forestales y Ambientales, Mérida, Venezuela.* forest.ula.ve
[6]*Universidade Federal de São João del-Rei (UFSJ), São João del Rei, MG, Brazil.* ufsj.edu.br
[7]*Universidad Autónoma de Yucatán, Mérida, Mexico.* uady.mx

Abstract

Leucaena leucocephala (leucaena) is native to Mexico and Central America and is currently naturalized in the majority of Latin American countries. Over the last 2 decades, considerable research and promotion of leucaena have been carried out in Colombia, Mexico, Cuba, Brazil, Paraguay and Argentina. Research focused on the agronomic and management options for feeding beef, dairy or dual-purpose animals, with some studies on germplasm, weediness issues, toxicity, organic fertilizer application and environmental services.

Over the past 10–15 years, establishment and management of leucaena feeding systems in Latin America have varied according to country. For instance, intensive Silvopastoral Systems (iSPS) models are widely promoted and successfully adopted in Colombia, Mexico, Cuba, Venezuela and Northeast Brazil. In iSPS, leucaena is planted at high density (>10,000 trees/ha), in combination with improved tropical grass and high-value timber species (200–400 trees/ha), and intensively managed employing rotational grazing.

In Paraguay and Argentina, leucaena is planted in single or double hedgerows with inter-row alleys of 6–8 m, following the configuration used in Australia and mainly focused on beef production. In Mexico, leucaena is also cultivated with *Tithonia diversifolia* or lemon trees. Meanwhile, in other countries such as Cuba, leucaena has been established as protein banks using single/twin rows with inter-row spacing of 2–4 m for feeding beef, dairy or dual-purpose animals. Overall, paddock sizes for protein banks and iSPS range between 0.3 and 50 ha, while single and twin hedgerow systems are generally established over larger areas (20–500 ha). Despite the significant benefits demonstrated by research on leucaena feeding systems over the past 2 decades, coupled with successful outcomes for farmers who have adopted these systems, total area sown remains low across Latin America. This review provides a comparison between Latin American and Australian leucaena pasture systems, and recommendations for future collaborative research between countries.

Keywords: Adoption, beef production, dairy production, high-quality forage, tree legumes.

Resumen

Leucaena leucocephala (leucaena) es una especie leguminosa nativa de México y América Central y actualmente se la puede encontrar naturalizada en la mayoría de los países de América Latina. En las últimas dos décadas, considerables avances en investigación y promoción de leucaena se llevaron a cabo en Colombia, México, Cuba, Brasil, Paraguay y Argentina. Estas investigaciones se enfocaron en el manejo agronómico de la especie y su uso para la alimentación del

Correspondence: Nahuel A. Pachas, School of Agriculture and Food Sciences, The University of Queensland, St Lucia Campus, Brisbane, QLD 4072, Australia. E-mail: a.pachas@uq.edu.au

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ganado de carne, leche o doble propósito, mejoramiento genético, toxicidad, fertilización orgánica y servicios ambientales.

Sin embargo, el establecimiento y manejo de leucaena en América Latina son diferentes según región. Por ejemplo, el modelo de sistemas silvopastoriles intensivos (iSPS) es ampliamente promovido y exitosamente adoptado en Colombia, México, Cuba, Venezuela y el noreste de Brasil. En estos sistemas, leucaena es plantada en altas densidades (>10,000 plantas/ha), en combinación con gramíneas mejoradas y especies maderables de alto valor (200−400 árboles/ha). En Paraguay y Argentina, leucaena es plantada en hileras simples o dobles dejando callejones entre 6 y 8 m, donde se siembran gramíneas. Este modelo es muy similar al utilizado en Australia y su uso principal es para la alimentación de ganado de carne. En México, esta especie se puede encontrar asociada con *Tithonia diversifolia* o con árboles frutales (p.ej. cítricos). En otros países, leucaena es usada como banco de proteína y plantada en hileras simples o dobles con callejones de 2−4 m para alimentar ganado de carne, leche o doble propósito.

Los bancos de proteína y los iSPS se encuentran establecidos en áreas entre 0.3 y 50 ha, mientras que los sistemas de hilera simple o dobles asociados con gramíneas se encuentran plantados en grandes extensiones (20−500 ha). A pesar del gran avance en investigación y promoción de esta especie en las últimas décadas, su adopción es aún baja en América Latina. Este trabajo presenta información sobre el manejo y establecimiento de leucaena en América Latina, una comparación con el sistema australiano y recomendaciones de trabajos colaborativos entre países.

Palabras clave: Adopción, arboles leguminosos, forrajes de alta calidad, producción de carne, producción de leche.

Introduction

Leucaena leucocephala (leucaena) is a native multipurpose tree legume from Mexico and Central America and is currently naturalized in the majority of Latin American countries. Its use varies widely from human food, fuel, timber and shade (for perennial crops and livestock) to fodder for ruminant animals. According to Parrotta (1992), this species can be found in a range of environmental conditions across latitudes between 30° N and 30° S. It grows well in areas that receive annual rainfall of 500–2,000 mm, with dry seasons of 2–3 months, and with well-drained soils that are slightly acid (pH 6) to moderately alkaline (pH 7.5). Optimal temperatures range between 20 and 30 °C, but its growth is restricted at low temperatures (<15 °C), although it can survive mild frost events.

The aim of this review is to provide an update of research carried out with leucaena in the last 18 years (2000–2018) in Latin America and describe its establishment and management for ruminant feeding by region. Finally, it will provide a comparison between leucaena feeding systems in Latin America and Australia.

Research in Latin America

In the past 2 decades, Latin American researchers have actively participated in various leucaena studies involving germplasm, weediness issues, toxicity, animal production, organic fertilizer application and environmental services. To estimate the level of research activities on this species, Scopus, the worldwide largest abstracting service and database of peer-reviewed research literature (scientific journals and books plus congress and conference proceedings), was used to count the number of leucaena publications (where leucaena was included in the title or keywords) carried out by Latin American institutions. From 441 papers published between 2000 and 2018, 95% involved researchers affiliated with institutions from Brazil (117), Mexico (111), Cuba (89), Venezuela (44), Colombia (40) and Argentina (14) (Figure 1). Although it may not take into account all research published in congress and conference proceedings, it provides a snapshot of the status of leucaena research in Central and South American countries (Figure 1, Table 1).

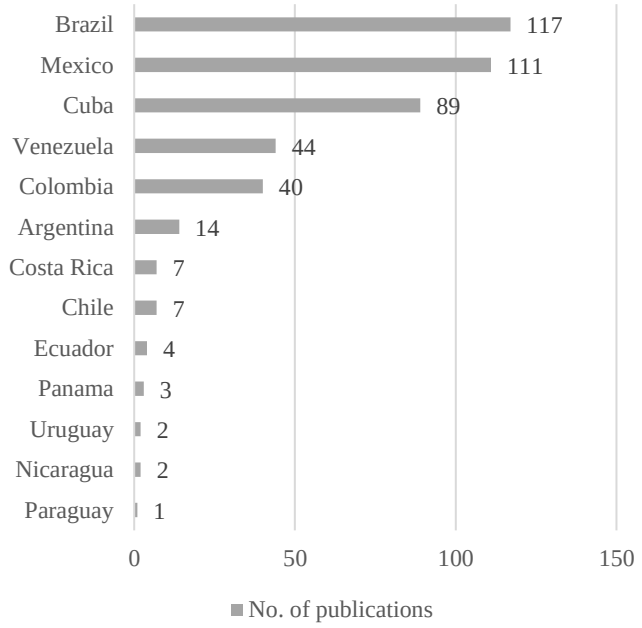

Figure 1. Total publications regarding leucaena by country.

Table 1. Top 3 Latin American institutions in each country during 2000–2018, listed by Scopus.

Country	Top 3 institutions
Brazil	• Empresa Brasileira de Pesquisa Agropecuária (Embrapa) • Universidade Estadual Paulista (UNESP) • Universidade de São Paulo (USP)
Mexico	• Universidad Autónoma de Yucatán • Universidad Nacional Autónoma de México • Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP)
Cuba	• Instituto de Ciencia Animal (ICA) • Estación Experimental de Pastos y Forrajes Indio Hatuey • Centro Nacional de Sanidad Agropecuaria
Venezuela	• Instituto Nacional de Investigaciones Agrícolas (INIA) • Universidad de los Andes (ULA) • Universidad del Zulia (LUZ)
Colombia	• Centro para la Investigación en Sistemas Sostenibles de Producción Agropecuaria (CIPAV) • Universidad Nacional de Colombia • International Center for Tropical Agriculture (CIAT)
Argentina	• Instituto Nacional de Tecnología Agropecuaria (INTA) • Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) • Universidad de la Plata

Establishment and management of leucaena by region

Leucaena grows well and is adapted to a wide variety of environments in Central and South America (Lascano et al. 1995). However, its establishment and management vary by region. In this review, we have identified and characterized 3 different systems for growing and utilizing leucaena:

- Protein bank systems: mainly found in Cuba and Venezuela;
- Intensive Silvopastoral Systems (iSPS): found in Colombia (Caribbean region, Inter-Andean valleys); Mexico (Michoacán, San Luis de Potosí – Huasteca region, Veracruz, Tamaulipas, Yucatán, Campeche, Jalisco, Guerrero and Colima states); Panama (Azuero province); Cuba; Venezuela (Llanos); Brazil (state of Maranhão); and Argentina (Misiones province); and
- Australian-style systems: found in Paraguay (Chaco) and Argentina (Chaco, Formosa and Corrientes provinces).

A brief description of each production system is as follows:

Protein bank systems

In this system, leucaena is established strategically in small areas of the farm with the aim of providing a high-level protein source for cattle. Typically, site preparation is carried out by strip or total cultivation of the paddock using animal draft power and to a lesser extent, if farmers have access to tractors, mechanical cultivation. Inoculation of seeds with specific *Rhizobium* strains, such as ICA 4006 and ICA 4010 developed by the Institute of Animal Science (ICA, Cuba), is recommended. Fertilizer application is not a common practice owing to its high cost and labor requirement; however, the use of animal manure is highly recommended in soils that have been previously used for cropping. According to Ruiz et al. (1989), the optimal time for establishing leucaena is during the rainy season (April–June), particularly for cvv. Peru and Cunningham. Planting configuration of leucaena is usually in single or twin rows (2–5 plants/m), with 3–4 m inter-row spacing, providing densities between 5,000 and 8,000 shrubs/ha. Chemical or mechanical weed control is critical during the first 2–3 months after planting. Leucaena is intercropped with grasses such as *Cynodon nlemfuensis* and *Megathyrsus maximus* once the leucaena seedlings reach 0.1 m height. However, first grazing or cutting is not recommended until leucaena plants reach 1.2–1.5 m height. The main use of the protein bank is for feeding dairy and beef cattle in cut-and-carry systems or for grazing with limited temporal access.

Intensive Silvopastoral Systems (iSPS)

ISPS were developed in Colombia by CIPAV (Centro para la Investigación en Sistemas Sostenibles de Producción Agropecuaria). In these systems, leucaena is planted at high density (>10,000 shrubs/ha) in combination with improved grasses, including native or exotic trees and palms (200–400 trees/ha). The establishment, management and benefits of iSPS have been described and reported (Murgueitio and Ibrahim 2008; Uribe et al. 2011; Murgueitio et al. 2011, 2016; Zapata Cadavid and Tapasco 2016). Briefly, land preparation is carried out by mechanical cultivation and the needs for fertilizer application or soil amendment are assessed according to soil analysis. Common leucaena cultivar used is Cunningham. Before planting, seeds are scarified and inoculated with specific *Rhizobium* at 0.5 kg bacterial culture/10 kg seeds. Recommended planting configuration is single rows with 1.5–1.6 m between rows and 0.3–0.4 m between plants within rows, which provides leucaena densities of 10,000–22,000 shrubs/ha. Improved grasses such as *Megathyrsus maximus* and *Cynodon plectostachyus* are sown at the same time as leucaena or about 45 days after planting, when the legume has established. Overall, the first

grazing is carried out 3–6 months after planting leucaena using a low stocking rate, followed by a pruning of leucaena at 0.5–0.75 m for stimulating growth of branches. Leucaena-grass pasture is managed using intensive rotational grazing (12–48 hours) with electric fences and permanent supply of water, followed by 40–45 days for pasture recovery. The main use of iSPS is for feeding beef cattle, dual-purpose cattle and cows on tropical dairy farms (Figures 2 and 3). It is important to mention that leucaena toxicity is not considered as a limitation; therefore animal inoculation with the rumen bacterium *Synergistes jonesii* is not a common practice

Figure 2. Intensive silvopastoral system with leucaena - *Cynodon plectostachyus* pasture in the tropical dairy system at El Hatico, Valle del Cauca, Colombia.

Figure 3. Intensive silvopastoral system with *Leucaena leucocephala* and *Megathyrsus maximus* at Apatzingán, Michoacán, Mexico. Some leucaena is allowed to grow tall to provide shade.

Adopted Australian-style systems

In these systems, adopted and adapted by Paraguayan and Argentinian farmers, leucaena-grass pastures are established following procedures similar to those recommended in Australia (Dalzell et al. 2006; Radrizzani et al. 2019). Briefly, land is prepared by repeated mechanical cultivation and seed is scarified and inoculated with specific *Rhizobium* cultures. Common cultivars used are Cunningham, Tarramba and Peru. Recommended configuration is single or twin rows (1 m apart) with inter-row alleys between 5 and 6 m. After planting, weeds are controlled by chemical or mechanical means and, to a lesser extent, using manual weeding. In Argentina and Paraguay, intercropping with maize, soybean or sorghum is practiced after planting leucaena with the aim of controlling weeds and making use of the inter-row alleys. Grasses are introduced when leucaena reaches 1 m height (Figure 4). The common companion grasses are *Megathyrsus maximus*, *Chloris gayana* and *Urochloa brizantha* (cvv. Marandu, Xaraés and Mulato II). Leucaena is grazed initially when it reaches 2–2.5 m height. After that, the pasture is managed using rotational grazing mainly by beef cattle. Paraguayan producers inoculate their animals with rumen fluid, considered to contain the bacterium *Synergistes jonesii*, but this practice is not carried out in Argentina due to unavailability of inoculum. Still, the occurrence and effect of toxicity on animal productivity are unclear. Radrizzani and Nazca (2014) reported animal toxicity symptoms in an experiment with high proportion of leucaena in available feed (40%) in beef cattle in the Chaco region, Argentina. However, in several other experiments animals showed no symptoms (Gándara et al. 1986; Lacorte et al. 1987; Gándara and Casco 1993; Lacorte 2001; Pachas et al. 2012).

Figure 4. Leucaena intercropped with sorghum for silage in Pampa del Infierno, Chaco, Argentina.

Comparison between the Latin American and Australian leucaena feeding systems

Leucaena pasture systems used in Latin America and Australia have similarities and differences, which are summarized in Table 2. Regardless of differences, e.g. planting density and establishment, both systems are highly productive, profitable and sustainable (Klassen 2005; Dalzell et al. 2006; Murgueitio et al. 2011; Calle et al. 2013; Pachas et al. 2018).

Table 2. Summary of similarities and differences of leucaena feeding systems in Latin America and Australia.

Variable	Latin America	Australia
Planting density	Leucaena is planted at high density (>10,000 shrubs/ha) in iSPS, ~5,000–8,000 shrubs/ha in protein banks and lower density in the Argentinean and Paraguayan Chaco (3,000–8,000 shrubs/ha).	Leucaena is planted at lower density (3,000–8,000 shrubs/ha).
Planting configuration	Multi-strata systems (at least 3 strata): improved grasses, leucaena and timber trees, palms/fruit trees) in iSPS; otherwise only 2 strata using improved grasses.	Two strata: improved grass and leucaena.
Establishment	Grass is sown early (0–3 months) after planting leucaena. In the Argentinian and Paraguayan Chaco region it is common to intercrop the alleys with annual crops.	Grass is established once leucaena is fully established (8–12 months) to avoid competition. Meanwhile, weeds are chemically and mechanically controlled.
Uses	Dairy and beef production	Beef production
Area per farmer	Farmers plant leucaena on a small scale (1–50 ha).	Farmers plant leucaena on a large scale (50–6,000 ha).
Results	High animal productivity	High animal productivity

An important difference between the regions is that leucaena is an important component in the diet of dairy cattle in South and Central America but is used only to feed beef cattle in Australia. Another difference between regions is the area cultivated. The area of leucaena in Latin America is not well defined but it is most likely to be between 45,000 and 55,000 ha. The area established in Cuba is approximately 20,000 ha, ~7,000 ha as protein banks and the remaining 13,000 ha in association with grasses (T. Ruiz unpublished data). There are approx-imately 12,000 ha in 10 states of Mexico (Ramírez-Avilés et al. 2019), ~10,000 ha in Paraguay (Glatzle et al. 2019), with 3,000–5,000 ha in Venezuela (E. Escalante unpublished data), ~2,400 ha in Argentina (Radrizzani et al. 2019), 3,000 ha in Colombia (F. Uribe unpublished data) and perhaps 1,000–1,500 ha in the remainder of Latin American countries. This figure is significantly lower than ~200,000 ha cultivated in Australia. However, due to leucaena being planted in small areas (1–50 ha), the number of smallholders who have adopted this system is relatively higher (estimated at ~6,000–8,000 producers) compared with Australia (estimated at 500–1,000 producers).

Conclusions

Over the last 2 decades, significant research effort on leucaena feeding systems has occurred in Latin America with successful adoption in Colombia, Mexico, Cuba, Venezuela, Paraguay and Argentina. The research has shown that leucaena systems are highly productive and adapted to a wide range of environmental conditions. However, adoption of this technology remains low in cattle-producing countries. The similarities and differences between Latin American and Australian leucaena systems can be viewed as an opportunity for future collaboration between regions. For instance, Australia has developed a larger number of cultivars, which can be used in Latin America; meanwhile, although several germplasm collections were carried out in South and Central America, no successful cultivars have been released to the market. The multi-strata configuration systems used in South America´s iSPS have shown environmental benefits and we consider they should be tested in Australia, with the aim of improving carbon sequestration and biodiversity. The use of leucaena for feeding dairy cattle in subtropical environments in Australia could also be tested. Finally, collaboration between Central and South American researchers and producers should be promoted and fostered with the aim of enhancing adoption of this species and collaboration between countries.

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ILC2018 Poster and Producer paper*

Leucaena establishment on frontage country in the Queensland Gulf

Establecimiento de leucaena en el norte de Queensland, Australia

JOE ROLFE[1], CRAIG LEMIN[1], BERNIE ENGLISH[1], ROBERT CAIRD[1], EMMA BLACK[2], LINDSEY PERRY[3], RONNY & COLLEEN HENRY[4] AND GLEN & CHERYL CONNOLLY[5]

[1]*Queensland Department of Agriculture and Fisheries, Mareeba, QLD, Australia.* daf.qld.gov.au
[2]*Queensland Department of Agriculture and Fisheries, South Johnstone, QLD, Australia*. daf.qld.gov.au
[3]*Queensland Department of Agriculture and Fisheries, Cloncurry, QLD, Australia.* daf.qld.gov.au
[4]*Riverview Station, Georgetown, QLD, Australia*
[5]*Blanncourt Station, Georgetown, QLD, Australia*

Keywords: Basalt soils, frontage country, grazing, tree legumes, Wondergraze.

Introduction

Introduction and successful establishment of leucaena (*Leucaena leucocephala*) has the potential to improve annual liveweight gains (LWGs) of grazing cattle in northern Australia, sustainably increase gross margins and mitigate methane production (Harrison et al. 2015). However, leucaena adoption in northern Queensland to date has been low (<2,500 ha established) compared with other regions of the State.

Impediments to leucaena adoption in north Queensland include: (i) poor producer awareness of the productivity benefits of leucaena; (ii) a lack of farming expertise and limited access to suitable machinery; (iii) high establishment costs; (iv) occurrence of psyllid infestations and subsequent leucaena productivity losses; and (v) landscape constraints including low soil fertility, poor soil drainage and standing native timber. This paper describes the efforts of 2 producers to establish leucaena on cleared country adjacent to the Gilbert River (frontage country) in the Northern Gulf Region of Queensland.

Materials and Methods

Leucaena was established recently on 2 family-run breeding enterprises (Blanncourt and Riverview Stations), west of Georgetown in the Queensland Gulf Region, on previously cleared alluvial soils adjacent to the Gilbert River. Background enterprise details are outlined in Table 1 and both operations can be characterized as being well managed by progressive producers with previous farming experience and access to machinery. At Blanncourt, 54 ha of leucaena was established during the 2015/16 wet season (after a failed attempt the previous year), while 160 ha was sown at Riverview during the 2017/18 wet season.

At Blanncourt, the paddock was deep-ripped in October 2014 and 2 m cultivated strips prepared at 10 m spacing. Superphosphate (8% P, 11% S) was applied (250 kg/ha) along the whole strip. Wondergraze was sown (1.5 kg/ha) in single rows in the 2014/15 wet season (December) after 55 mm rainfall. Spinnaker® (700 g/kg imazethapyr) was applied at the time of sowing at 70 g/ha. Verdict® (520 g/L haloxyfop) at 300 mL/ha was used for post-emergent grass control. Dictate (480 g/L bentazone) at 2 L/ha was also used on some rows for broad-leaf weed control. Plants on less than 5 ha survived due to strong competition from nut grass (*Cyperus esculentus*) and low rainfall (only ~250 mm received over the wet season). The majority of the site required re-sowing due to poor emergence and lack of follow-up rain. In the 2015/16 year, plant strips were cutter-barred (bulldozer rippers inserted in ground and a connecting bar between the ripper boots severs woody regrowth and nutgrass roots and rhizomes to a depth of 300 mm approx.) in September. While this was effective, nitrogen mineralization from cutter-barring encouraged heavy broad-leaf weed growth. Cultivation of the strips with disc and tyned implements over the October/November period was used for weed control. Rainfall (75 mm) in December 2015 allowed planting in late December, with Spinnaker® (100 g/ha) again applied as a pre-emergent weedicide and

Correspondence: J. Rolfe, Queensland Department of Agriculture and Fisheries, Mareeba, QLD 4880, Australia.
Email: joe.rolfe@daf.qld.gov.au

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Table 1. Enterprise characteristics of properties where leucaena has been established on Gilbert River frontage country.

	Blanncourt Station	Riverview Station
Annual average rainfall (mm)	800	800
Elevation (masl)	~200	~200
Location	Georgetown district, Northern Gulf	Georgetown district, Northern Gulf
Area (ha)	18,750	7,300 plus agistment on neighboring property of 13,000 ha
Soils (for leucaena)	Sandy-loam alluvials (Gilbert River frontage)	Sandy-loam alluvials (Gilbert River frontage)
Typical soil phosphorus levels (Colwell mg/kg)	28	18
Typical soil sulphur levels (sulphate, mg/kg)	1.6	2.5
Stocking rate	1 AE to 8–10 ha	1 AE to 8–10 ha
Estimated stocking rates on frontage with improved pastures and leucaena	1 AE to 3 ha	1 AE to 3 ha
Markets	Abattoirs, live export, feedlots, local store sales	Mainly private sale (buyers send to live export or fattening markets)

AE = animal equivalent of ~450 kg steer.

Dictate® (2 L/ha) was used for broad-leaf weed control after leucaena emergence. Verdict® (300 mL/ha) was also applied to control grasses, although 2 additional cultivations were performed after sowing. Granulated sulphur (90% S; 50 kg/ha) was applied over the plant rows once establishment was assured.

At Riverview, the paddock was cutter-barred in September 2017 to control nut grass and woody weeds and several subsequent cultivations developed a fine seedbed. Superphosphate (250 kg/ha) and granulated sulphur (70 kg/ha) were applied over 3 m of the planting strip. Unusually heavy rain (120 mm) in October negated the effectiveness of the cutter-barring and re-invigorated growth of nut grass. The December/January period was then hot (>35°C) and dry. After re-cultivating in December, some planting was possible in January 2018 but was compromised by competition from weeds and set-up issues with a new twin-row planter. Sowing was done mostly in February, with 150 ha of cv. Wondergraze and 10 ha of cv. Redlands planted at 1.5 kg/ha seeding rate. Immediately after sowing, Vezir® (700 g/kg imazethapyr) at 140 g/ha and glyphosate (570 g/L) at 2 L/ha were applied in a mix for knockdown and pre-emergent weed control. Persistently wet conditions prevented timely weed control post-sowing. Cultivation was not possible but Sempra® (750g/kg halosulfuron-methyl) at 100 g/ha and Verdict® at 300 mL/ha combined with a wetting agent (Banjo®, 725 g/L methyl ester) were applied as a mix during March. By April problems were being experienced in some areas with termites eating young leucaena stems. A ground-based application of Regent® (200 g/L fipronil) at 100 mL/ha was made over the plant rows in an attempt to control termites.

Results

Overall establishment success at Blanncourt was ~75% across the paddock as a whole after the second year planting. Soils varied across one end of the paddock with heavier clay loams in low areas. Leucaena germination was usually satisfactory in these areas but emergence or subsequent establishment was poor due to soil surface crusting, poor drainage and heavy weed competition. Leucaena establishment across the remainder of the paddock (more even loamy soils with better drainage) was very favorable. The pre-emergent herbicide applied at sowing was ineffective, although grasses were well controlled with Verdict®. Cattle were introduced to the paddock in July 2016, when leucaena was about 2 m high. No animal performance data have been collected to date.

At Riverview, establishment success was approximately 65% across the paddock as a whole. Some areas of the paddock had full establishment, while other areas had patchy establishment due to variable soil characteristics, seed blockage issues during sowing and termite damage post-planting. The herbicide mix of glyphosate and Vezir® applied at sowing was only partially effective as both a knock-down and pre-emergent treatment due to the hot and dry conditions. Persistently wet conditions later in February then prevented timely weed control, leading to strong competition with young leucaena in many areas. The mixed herbicide application in March caused some yellowing of leucaena (leaf) but it recovered within a few weeks. This was attributed to the Sempra®, as such damage has not previously been observed with Verdict®. Cattle commenced grazing in August 2018 (232 head averaging 229 kg on the 160 ha). No animal performance data have been collected to date.

Figure 1. Leucaena planted in single strip row on Blanncourt (left) and Ronny and Colleen Henry with a double-row planting (right) on Riverview station.

Figure 2. Glen and Cheryl Connolly, Blanncourt Station (left) and leucaena establishment neighbor day at Blanncourt in 2016 (right).

Discussion and Conclusions

While these areas are limited in extent, establishing leucaena on cleared frontage country in northern Queensland is a significant opportunity for producers with access to this land type to improve productivity. Fertility limitations of these soils can be overcome economically with fertilizer applications but weed control and climate variability remain significant challenges to reliable leucaena establishment. Experiences at Blanncourt and Riverview have highlighted ongoing issues with regard to effective weed and pest control. Some options for improving weed control include slashing or mowing and increased cultivation (post-sowing), increased rates of pre-emergent herbicide (risky) and establishing buffel first (to out-compete broad-leaf weeds), then sowing leucaena into sprayed-out or cultivated strips. Pests (grasshoppers, termites and wallabies) can exert significant pressure on young leucaena plants and are unpredictable and expensive to control.

Nonetheless, successful establishment at Blanncourt and Riverview has demonstrated that motivated producers can successfully establish leucaena on frontage country in north Queensland (Figures 1 and 2). Owners of both enterprises are planning to establish a further 600 ha of leucaena over the 2018/19 wet season.

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ILC2018 Keynote paper*

Review of establishment practices of *Leucaena leucocephala* cv. Tarramba in West Timor, Indonesia

Revisión de prácticas de establecimiento de Leucaena leucocephala *cv. Tarramba en Timor Occidental, Indonesia*

JACOB NULIK AND DEBORA KANA HAU

The East Nusa Tenggara Assessment Institute for Agriculture Technology, Kupang, Indonesia. ntt.litbang.pertanian.go.id

Abstract

With increasing cattle production in East Nusa Tenggara Province there is an urgent need to increase plantings of high quality forage such as Tarramba leucaena. This requires stakeholders to acquire knowledge and practical skills to achieve reliable plant establishment. As part of a study of Tarramba leucaena adoption in East Nusa Tenggara, it became clear that the best method to establish leucaena was by transplanting 1–2-month-old seedlings at the beginning of the rainy season that had been pre-prepared in poly-bags at a nursery. However, with varied conditions at the study locations, such as the absence of a dry season water source, farmers have used other methods, including: direct seeding; poly-bag seedlings planted later in the wet season; or older bare-root seedlings harvested from a high-density nursery or from volunteer seedlings growing between rows of established leucaena. This paper elaborates on the different methods of establishment in farmer plantings in Kupang District (West Timor region of East Nusa Tenggara Province), Indonesia.

Keywords: Bare-root seedlings, establishment, tree legumes.

Resumen

En vista del incremento de la producción de ganado en la provincia de Nusa Tenggara Oriental existe una necesidad urgente de aumentar la producción de forraje de alta calidad, por ejemplo de la leucaena cv. Tarramba. Esto requiere conocimientos y habilidades prácticas de los productores para poder lograr un establecimiento confiable del cultivo. Un estudio de adopción de la leucaena Tarramba en Nusa Tenggara Oriental mostró que el mejor método para establecer la leucaena fue el trasplante de plántulas de 1–2 meses de edad al comienzo de la época de lluvias, usando plántulas en bolsas de polietileno procedentes de un vivero. Sin embargo, en vista de la variabilidad de las condiciones de establecimiento en los sitios de estudio, tales como la disponibilidad de agua en la época seca, los productores usan diferentes métodos, entre ellos: siembra directa; trasplante de plántulas en bolsas plásticas más tarde en la época lluviosa; o trasplante de plantas pequeñas, menos jóvenes con las partes aéreas recortadas, cosechadas en un vivero de alta densidad u obtenidas de poblaciones espontáneas de leucaena que aparecen entre las hileras de árboles en producción. Este documento describe y analiza los diferentes métodos de establecimiento usados por los productores en el distrito de Kupang (región de Timor Occidental, provincia de Nusa Tenggara Oriental), Indonesia.

Palabras clave: Establecimiento, leguminosas arbóreas.

Correspondence: Jacob Nulik, East Nusa Tenggara Assessment Institute for Agriculture Technology, Kupang, Nusa Tenggara Timur, Indonesia. Email: Jacob_nulik@yahoo.com

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Introduction

Adoption of the drought-resistant high-quality forage tree legume leucaena (*Leucaena leucocephala* cv. Tarramba) for cattle fattening is increasingly common in eastern Indonesia, especially in the Fatuleu subdistricts of West Timor. Successful establishment of Tarramba leucaena requires good knowledge of its establishment needs as well as practical skills. Earlier studies (Nulik et al. 2013) showed that the most successful method was planting of seeds in poly-bags 1–2 months before transplanting seedlings into the field once the rainy season had commenced in November–January. However, as the practice of planting Tarramba leucaena expanded, so did variation in site conditions. For instance, the Tunas Muda farmer group (Bilboto hamlet, Camplong II village, Fatuleu subdistrict), located in an area where availability of water was severely restricted during the dry season, waited until the early rainy season before poly-bag seedlings could be prepared for transplanting during February-March.

A review of modified establishment methods was conducted on several project sites where many new farmers had planted leucaena in West Timor, especially in Oebola Dalam and Camplong II villages in Fatuleu subdistrict, and in Nunsaen village in Central Fatuleu subdistrict. The objective was to observe how farmers had adapted their planting techniques to suit the various climatic and edaphic conditions and still achieve successful establishment of Tarramba leucaena.

Climate and soils of eastern Indonesia

Timor Island has a tropical wet and dry savanna climate (Köppen-Geiger classification: Aw) with a pronounced dry season. West Timor is characterized by a tropical monsoonal climate with erratic rainfall patterns (Table 1), often leading to plant establishment failures (Nulik 1994) even when establishment practices may have been conducted appropriately.

Timor Island was formed from coral uplift, and thus the main parent material of the soils is limestone rock. This parent material has led to the formation of 2 main soil types, black (Mollisol) and red (Alfisol) soils (Mella and Mermut 2010) (Figure 1). Nulik et al. (2013) reported that the black sediment soils (black clays and sandy clays) gave the best early plant growth during the establishment of Tarramba leucaena.

Current establishment techniques

Unlike Amarasi district in West Timor (Piggin and Nulik 2005), the Fatuleu region traditionally involved free grazing of breeding cows on communal pastures, where farmers produced calves for sale but suffered high calf mortality and consequently low weaning outcomes (Dahlanuddin et al. 2019). Moreover, with the increasing human and livestock populations, especially cattle, degradation of native pastures has become significant with extensive invasion by the unpalatable weed *Chromolaena odorata* (Figure 2). The introduction of Tarramba leucaena into the region was deemed the best solution to improve the livelihoods of poor farmers (Dahlanuddin et al. 2019) by greatly increasing productivity of their cattle herds, and therefore cash flow to families, while simultaneously controlling the invasion of the unpalatable *Chromolaena*.

Thus expansion of programs to foster adoption of the legume was encouraged. In response to differing site conditions we observed that farmers had modified their establishment techniques according to their particular farm situations, i.e. some prepared seedlings in poly-bags (Figures 3 and 4) for planting in the early wet season, some direct-seeded leucaena while sowing corn (Figure 5), while others used 'bare-root' planting material derived from seedlings or plants up to 2–3 years old (Table 2), which are dug out from under established tree rows and stripped of small branches and leaves. The benefits and problems associated with each establishment technique are described in Table 3.

Table 1. Climate of Kupang, West Timor.

Climate variable	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Annual
Average Max Temperature (°C)	31	32	33	33	33	32	31	31	31	32	32	31	32
Average Min Temperature (°C)	21	21	22	23	23	24	24	24	23	23	22	22	23
Average Precipitation (mm)	5	3	2	18	89	246	389	366	221	64	28	10	1441
No. of Wet Days (probability of rain on a given day; %)	1 (3)	1 (3)	1 (3)	4 (13)	8 (27)	15 (48)	17 (55)	17 (60)	11 (35)	7 (23)	4 (13)	2 (7)	88 (24)

Figure 1. Black Mollisols (left) and Red Alfisols (right), derived from limestone rock parent materials, are the main two soils in West Timor.

Figure 2. Overgrazed communal lands invaded by the unpalatable weed *Chromolaena odorata.*

Figure 4. Poly-bags planted with young Tarramba leucaena seedlings.

Figure 3. Village group preparing poly-bags for seeding with Tarramba leucaena.

Figure 5. Tarramba leucaena established following planting with corn.

Table 2. Establishment techniques for Tarramba leucaena in eastern Indonesia.

Village, Farmer Group	Soil type	Dominant weed, other establishment problems	Establishment technique	Area established (% of available land)
Oebola Dalam village				
Bersaudara	Red rocky soils (Alfisols)	*Chromolaena*, native grasses, free grazing animals	Seedlings established in poly-bags before rains	5% of 125 ha
	Black and Red soils (Mollisols and Alfisols)	Native grasses, free grazing animals	Direct seeding	20% of 125 ha
	Red rocky soils (Alfisols)	*Chromolaena*, native grasses, free grazing animals	Bare-root seedlings from 1–3 yr plants	75% of 125 ha
Camplong II village				
Setetes Madu	Black rocky soils (Mollisols)	*Chromolaena*	1–2 months before rain seedlings	80% of >50 ha
		Chromolaena	Early rain seedlings	20% of >50 ha
Talekomonit	Red and Black rocky soils (Alfisols and Mollisols)	*Chromolaena*	Direct seeding	70% of 60 ha
		Chromolaena	Early wet season seedlings in poly-bags	30% of 60 ha
Tunas Muda	Red and Black rocky soils (Alfisols and Mollisols)	Native grasses, *Chromolaena*	Direct seeding	40% of 30 ha
		Native grasses	Early wet season seedlings in poly-bags	60% of 30 ha
Sabu Bani	Red rocky soils (Alfisols)	Native grasses, *Chromolaena*	Early wet season seedlings in poly-bags	100% of 30 ha
Sanam Tuan	Black and Red rocky soils (Mollisols and Alfisols)	*Chromolaena*, native grasses	Early wet season seedlings in poly-bags	90% of 30 ha
			Direct seeding	10% of 30 ha
Nunsaen Village				
Amtoas	Black and Red soils (Mollisols and Alfisols)	Native grasses, *Chromolaena*	Before rain and early wet season seedlings in poly-bags	90% of 150 ha
			Direct seeding	10% of 150 ha

Table 3. The benefits and problems with various establishment techniques.

Establishment technique	Benefits	Problems
Prepared poly-bag seedlings 2–3 months before rainy season	• High establishment rate • Less competition with native grasses	• High labor demand • Need to buy poly-bags • Need dry season water source
Prepared poly-bag seedlings in early rainy season (November–January)	• No need for dry season water source • Reasonable establishment rate • Less competition with native grasses	• High labor demand • Need to buy poly-bags
Direct seeding	• Less labor required • No need to buy poly-bags • Planting can be done together with planting of corn	• Need proper weeding • Susceptible to free grazing animals and fire
Bare-root cuttings from seedlings and young plants under established trees	• Less labor required, no need to prepare seed bed • Good for controlling spread of leucaena plants outside established rows • Seedlings can be taken any time during the rainy season (can be 1–3-year-old seedlings) • Less competition from native grasses and weeds	• Need to transplant when rain is reasonably stable

Conclusions

We found that farmers modified their planting techniques for establishment of Tarramba leucaena in West Timor in response to conditions at specific locations. These modified planting methods included: (i) preparation of seedlings in poly-bags early in the rainy season (December–February); (ii) direct seeding with corn early in the rainy season; (iii) and planting of bare-root seedlings obtained from under the established tree rows. The last technique was successful in Oebola Dalam village. Nevertheless, the best outcome was confirmed as transplanting of pre-prepared poly-bag seedlings 2–3 months before the onset of the rainy season. The review also confirmed that plant growth was best on black soils derived from coral limestone soil (Mollisols) compared with growth on the red Alfisols in the region.

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ILC2018 Poster and Producer paper*

Leucaena as forage in northeast Africa

Leucaena como recurso forrajero en el noreste de Africa

ALAN ROBERTSON

Forage development consultant, Tenterfield, NSW, Australia

Keywords: Establishment, genetic material, tree legumes, utilization.

Introduction

Leucaena is naturalized throughout northeast Africa, and is continuing to spread. It is spectacularly productive in many areas, including Western Eritrea (Figure 1), the fertile mid-elevation areas of Ethiopia (Figure 2), parts of the Rift Valley, the shores of Lake Victoria and the flood plains of Somaliland and Somalia, with neutral to alkaline soil pH being a major factor in successful adaptation.

Figure 1. Leucaena with occasional irrigation, Western Eritrea.

Genetic material

The genetic material is diverse, from woody heavy-seeding types to those with excellent forage potential. Unfortunately, there has been some indiscriminate promotion of species, including *Leucaena trichandra* and *L. diversifolia*, on the basis of agronomic adaptation, without any cognizance of the importance of forage quality and therefore potential for livestock production. In addition, unsupervised seed collection has often resulted in a shift to material with inferior forage production. Promising cultivars, including Tarramba and Wondergraze, have been introduced. Major development programs can be based on these introduced cultivars or on the use of carefully selected naturalized material.

Figure 2. Leucaena spreading on roadsides in West Ethiopia.

Establishment

There are some major intensive initiatives encompassing leucaena. Ethiopia's Sustainable Land Management Program has generated many millions of seedlings in hundreds of government, communal and private nurseries, which also produce a wide array of other species, primarily for establishment on communally managed stock-exclusion areas, which are open to cut-and-carry management, but also on individual smallholdings. Some current programs in

Correspondence A. Robertson, 'Oakey Creek', Willson's Downfall via Tenterfield, NSW 2372, Australia.
Email: robertson.oaky@gmail.com

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Somalia and Kenya are promoting the intensive production of leucaena on smallholder farms, based on bare-root/bare-stem seedling production to generate seedlings 1–1.5 m tall. In most nurseries traditional approaches have generally been used, with production of small seedlings in tubes or bare-root systems. There are some current initiatives in broad-scale establishment on commercial farms, with direct seeding. However, even on these farms, given the very low labor costs, transplanting of large bare-root/bare-stem seedlings may be cost-effective, and would allow more-timely establishment, which is crucial with shorter growing seasons. There has been very little use of specific rhizobium inoculants.

Some other interventions have been undertaken. In the 1980s in Ethiopia, tonnes of seed were harvested at about US$0.15 per kg, with collection sites selected for high leaf production. This low seed cost enabled broad-scale seeding on miscellaneous sites including roadsides, aerial seeding on degraded slopes including limestone soils in the tributary gorges of the Blue Nile, sowing into sites heavily infested with weeds such as *Lantana camara* and inclusion in conventional pasture mixes. All programs resulted in successful establishment and persistence on suitable soils, and have contributed to continuing rapid colonization. Productivity has been constrained primarily by grass competition, which is not a major issue in most target areas; persistence under very heavy grazing pressure has generally been excellent.

Utilization

Historically, utilization has been sub-optimal, although leucaena's role in dry season feeding is widely recognized by smallholder farmers. With rapidly increasing land pressures, there is a shift towards more intensive utilization, and maintaining regularly-cut hedges. Farmers appreciate the additional benefits from the provision of shade and firewood. In some more-intensive systems, the role of leucaena in improvement of soil fertility in cropping systems is also recognized.

Other issues

Infestations of psyllids (*Heteropsylla cubana*) occur for only short periods in most areas, and do not justify any strong emphasis on the introduction of psyllid-tolerant genetic material.

There are no areas, currently, where the levels of use are likely to lead to mimosine toxicity. However, recent initiatives in promotion of more intensive systems will probably require greater attention to management of leucaena toxicity.

The future

Leucaena should be promoted much more widely throughout the region. There is no need for additional conventional research, although visual ranking of performance in wide-ranging environments should be routinely undertaken. Agencies (including ILRI and ICRAF) and other development groups need to ensure the promotion of superior material, and much greater care needs to be taken in local collection of seed, where emphasis must be on trees with high edible forage production. Accessions adapted to specific environments, including degraded sites with low rainfall, need to be selected and multiplied.

In intensive small-scale programs, bare-root/bare-stem nursery systems can be more widely used.

In most parts of the region, it is still feasible to produce seed at less than US$1/kg. Seed production programs should be initiated to provide large volumes of seed for use in diverse establishment systems, including direct seeding within livestock exclosures.

Leucaena should always be promoted in conjunction with other forage genetic material.

There is a major need for effective networking, with exchange visits to areas where leucaena is already playing a major role in livestock production and improved land management.

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A preliminary study of spatial distribution and plant density in a leucaena-grass planting in north Corrientes, Argentina

Estudio preliminar de la distribución espacial y densidad de plantas de leucaena en asociación con una gramínea en el norte de Corrientes, Argentina

LUIS GÁNDARA[1], MERCEDES M. PEREIRA[1] AND MARCOS STUP[2]

[1]*Instituto Nacional de Tecnología Agropecuaria (INTA), EEA Sombrerito, Corrientes, Argentina.* inta.gob.ar
[2]*Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste (UNNE), Corrientes, Argentina.* agr.unne.edu.ar

Keywords: *Brachiaria brizantha*, forage quality, forage yield, *Leucaena leucocephala***,** subtropics.

Introduction

In northeast Argentina, most beef cattle graze naturalized range pastures continuously, with limited winter supplementation and restricted access to improved pastures. Calving rates are low, averaging 40–50% annually and calf weaning weights average 150–170 kg at 6 months. Overall, productivity remains low (30–40 kg LW/ha/yr), mainly due to poor cattle nutrition (Goldfarb et al. 1993; Goldfarb and Casco 1994). In past decades, several improved grass and legume species were evaluated as a strategy to overcome this problem (Goldfarb et al. 1993; Goldfarb and Casco 1998) with *Leucaena leucocephala* (leucaena) showing definite potential. It was introduced in the 1970s into Corrientes Province and displayed good adaptation to the environmental conditions. When leucaena was evaluated as a protein bank and sown into natural grassland or established with a sown grasses, it has shown excellent potential by increasing productivity of these systems (Gándara et al. 1986; 1993). However, these evaluations were done using much lower densities of leucaena than recommended to maximize yield of leucaena (Pachas et al. 2018). Therefore, the objective of this study was to evaluate the effects of leucaena density, taking into account light interception, on both legume and total pasture yield and forage quality in a leucaena-grass pasture system.

Materials and Methods

Experimental site

The study was conducted at the National Agricultural Technology Institute (INTA EEA Corrientes) in Corrientes Province, Argentina (27º40´25.84 S, 58º45´13.59 W). The soil at the site is characterized as an Aquic Argiudol soil (pH: 5.9; OM: 1.93%; P: 2 ppm). Monthly rainfall recorded during the study period and monthly average temperature are presented in Table 1.

Experimental design

Leucaena (cv. Cunningham) was sown in October 2016 using a twin-row configuration (twin rows 1 m apart) with 2, 4 and 8 m spacings between the outer rows of the twin hedge-rows (treatments D-2, D-4 and D-8, respectively). Each hedge-row plot was 15 m long and 42 m wide. The experimental design was a randomized complete block with 3 replications. Leucaena was sown manually at a seeding density of 7–10 g per linear meter (objective: 10 plants/m of row). In this way, plant densities for D-2, D-4 and D-8 should be: 66,666, 40,000 and 22,222 plants/ha, respectively. In October 2017, leucaena plants were cut to 1 m height and *Brachiaria brizantha* cv. Marandú (brachiaria) was sown between hedge-rows at a seeding rate of 13.3, 8.0 and 4.443 kg/ha for D-2, D-4 and D-8, respectively.

Measurements

Accumulation of biomass of leucaena and brachiaria was measured in June 2018 (236 days after trimming in October 2017). Figure 1 provides an image of a D-2 plot at that time. Biomass of leucaena above 1 m was measured by harvesting subplots of leucaena (5 linear m of twin-row) and biomass of brachiaria above 10 cm (4 samples/treatment of 0.25 m^2). Before harvesting leucaena, average height, number of plants, shoots and branches of leucaena were measured.

Correspondence: Ing. Agr. Luis Gándara, Instituto Nacional de Tecnología Agropecuaria (INTA), EEA Sombrerito, Corrientes, Argentina. Email: gandara.luis@inta.gob.ar

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Table 1. Monthly rainfall (mm) and monthly average temperature (ºC) at INTA EEA Corrientes.

Year	Rainfall (mm)											
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
2016	172	95	122	355	12	210	28	86	19	285	126	320
2017	180	106	151	548	193	1	10	80	93	80	124	26
2018	329	52	231	0	220	26						
	Average temperature (ºC)											
2017	27.4	26.1	24.7	20.2	18.9	17.7	17.9	18.6	20.4	21.4	2.8	27.4

Then, leucaena was cut to 1 m above ground level and biomass partitioned into edible biomass (leaves, tender/ herbaceous stems <6 mm in diameter) and lignified/ woody stems (>6 mm in diameter).

Figure 1. Treatment D-2 (2 m distance between twin hedgerows) of leucaena with brachiaria.

Dry matter concentration of both leucaena and brachiaria was determined by drying fresh material (subsamples) in an oven at 65–70 ºC for 72 h to constant weight. For each treatment, representative subsamples (200 g of fresh biomass) of leucaena and brachiaria were selected and taken to the lab for determining N concentration by the Kjeldahl method. Percentage of crude protein (CP = N × 6.25) of leucaena-grass pasture for each treatment was then determined by weighting its contribution according to the proportion of leucaena and grass biomass (t DM/ha).

Pasture photosynthetically active radiation interception (light interception - LI) was determined by measuring incident light in the open sky (OS) and within the inter-rows (IR) with a ceptometer (Cavadevice, Buenos Aires, Argentina) and was expressed as percentage of shade using the following expression: shade % = 100 (OS – IR). LI measurements were taken on a sunny day between 11:00 h and 13:00 h in February 2018. Thirty measurements were recorded within each plot by placing the ceptometer along an equidistant transect between the middle points of the inter-rows.

Statistical analysis

Above-ground biomass, edible biomass, proportion of grass and legume, CP concentration and light interception (shade) were analyzed by ANOVA, and means were compared by the Tukey test (P<0.05). Statistical analysis was carried out using InfoStat® software.

Results

The numbers of leucaena plants/m, shoots per leucaena plant and height of leucaena plants did not differ significantly between treatments. Average numbers of leucaena plants per linear meter of individual rows were 9.1 plants/m, with 20.5 primary shoots/m and 2.1 m height. The levels of shading increased as spacings decreased (P<0.05). Average values of the measured variables are shown in Table 2.

Table 2. Total accumulated biomass of leucaena (L) and brachiaria (G), edible biomass (L+G), proportions of legume and grass in edible biomass, crude protein (CP) of the edible forage and shade.

Treatment	Total biomass (t DM/ha)	Edible biomass (L+G) (t DM/ha)	Proportion of legume (%)	Proportion of grass (%)	CP (%)	Shade (%)
D-2	13.6a[1]	8.7a (6.2+2.5)	71a	29c	17.9a	82a
D-4	10.1b	7.8a (2.9+4.9)	38b	62b	14.4b	43b
D-8	9.2b	8.0a (1.2+6.8)	15c	85a	11.6c	23c

[1]Means followed by different letters within columns differ significantly at P<0.05.

Discussion and Conclusions

This study has provided valuable information on the vexing question of how far apart the twin rows of leucaena should be planted. As was expected, leucaena yield was related to initial planting density with highest yields occurring at the highest density, i.e. the narrowest inter-row spacing, while grass yield was inversely proportional to leucaena density. Interestingly, total edible forage from the leucaena-brachiaria pasture was independent of planting configuration, with only the proportion of legume and grass varying along with the inter-row spacing. At narrow inter-row spacing, grass biomass decreased, presumably due mainly to increased shading, and to a lesser extent to competition for nutrients and possibly water as high rainfall was registered during the experiment. This reduction of grass growth was compensated for by increased edible biomass of leucaena with the result that crude protein concentration of the available edible forage increased with higher densities of leucaena. One might expect that animal performance would benefit from the higher quality of the forage.

Pachas et al. (2018) also reported that higher biomass of leucaena and total biomass and reduced biomass of grass were associated with higher density of leucaena.

The high primary production obtained in this experiment suggests that animals grazing leucaena-grass pasture can be expected to achieve enhanced liveweight gains or that higher stocking rates can be maintained compared with unimproved grass pastures. Grazing studies are needed to confirm these hypotheses although Gándara et al. (1986) in a 2-year study showed a 171% increment in beef production (kg LW gain/ha/yr) when beef cattle grazed leucaena-grass pasture (*L. leucocephala* + *Digitaria decumbens*) compared with a naturalized pasture of *Sorghastrum agrostoides, Paspalum notatum, Paspalum plicatulum* and *Paspalum urvillei.*

The preliminary conclusion from this study is that narrower inter-row spacing will not reduce overall yield of edible forage but will increase the crude protein concentration of the forage under conditions similar to those in this study. Similar studies in a range of environments and a range of seasons are needed to confirm these preliminary findings. It is important to continue monitoring this experiment as we expect that growth of leucaena will increase relative to grass, which will be negatively impacted by increased shading and greater competition for water in drier years.

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ILC2018 Keynote paper*

An update on leucaena toxicity: Is inoculation with *Synergistes jonesii* necessary?

Una actualización sobre la toxicidad de leucaena: ¿Es necesaria la inoculación con Synergistes jonesii*?*

H. MAX SHELTON[1], GRAHAM L. KERVEN[1] AND SCOTT A. DALZELL[2]

[1]*School of Agriculture and Food Sciences, The University of Queensland, Brisbane, QLD, Australia.* agriculture.uq.edu.au
[2]*Leucaena Research and Consulting Pty Ltd, Port Macquarie, NSW, Australia.*

Abstract

Concern about mimosine toxicity and its management has contributed to the restricted adoption of leucaena as a forage for ruminants. The toxicity is a function of the antimitotic effects of mimosine, which is rapidly converted to isomers of hydroxypyridone (DHP), also toxic compounds, by plant and microbial enzymes. Work by R.J. Jones and colleagues (1960–1994) identified a rumen bacterium (*Synergistes jonesii*) capable of degrading DHP, and rumen fluid containing this bacterium was subsequently made available in Australia as a commercial inoculum for cattle producers.

Research by University of Queensland and CSIRO over 15 years, commencing in 2003, found evidence for another pathway of toxin management in Indonesia, where hundreds of Balinese farmers had fed uninoculated Bali bulls (*Bos javanicus*) up to 100% leucaena without experiencing toxicity symptoms, apart from an initial 1–2 week period while their cattle became adapted to the new diet. Tests showed that the Indonesian cattle were not degrading all DHP, as it appeared in high concentrations in urine samples, predominantly as 2,3-DHP and almost all (>97%) in a conjugated form. The conjugating compounds (glucuronic acid and sulfate compounds), produced in the liver, appeared to be the major pathway for neutralizing the toxicity of DHP. Other work revealed that *S. jonesii* was a ubiquitous organism in the rumen fluid of animals in all countries but always as a minor population, just detectable using new PCR-based assays, and sometimes not detected in all animals studied.

Since the Indonesian cattle fed leucaena suffered symptoms of mimosine toxicity for only a short time before quickly recovering, we hypothesize that conjugation of DHP by the liver was the major detoxification pathway for these animals. This detoxification pathway is also operative in Australia and other countries but further studies are needed to determine its significance.

Keywords: Conjugation, 'leucaena bug', microbial detoxification, ruminants, tree legumes.

Resumen

La preocupación sobre la toxicidad de la mimosina y su manejo ha contribuido a que la adopción de leucaena como forraje para los rumiantes estuviera restringida a nivel mundial. La toxicidad se debe a los efectos antimitóticos de la mimosina, la cual mediante enzimas microbianas y de la planta se convierte rápidamente en compuestos también tóxicos, isómeros de la hidroxipiridona (DHP). Los trabajos de R.J. Jones y sus colegas (1960–1994) identificaron una bacteria ruminal (*Synergistes jonesii*) que es capaz de degradar el DHP. Posteriormente el líquido ruminal conteniendo esta bacteria se convirtió en Australia en un inoculante comercial para los productores ganaderos.

En investigaciones realizadas por la Universidad de Queensland y CSIRO durante los últimos 15 años se encontró evidencia de otra vía de manejo de toxinas en Indonesia, donde cientos de productores balineses habían alimentado toretes no inoculados de ganado Bali (*Bos javanicus*) con hasta 100% de leucaena sin experimentar síntomas de toxicidad, aparte de un período inicial de 1–2 semanas durante el cual los animales se adaptaron a la nueva dieta. Las pruebas mostraron que el ganado

Correspondence: H.M. Shelton, School of Agriculture and Food Sciences, The University of Queensland, Brisbane, QLD 4072, Australia. Email: m.shelton@uq.edu.au

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indonesio no estaba degradando todo el DHP ya que aparecía en altas concentraciones en muestras de orina, predominantemente como 2,3-DHP y casi todo (>97%) en forma conjugada. Los compuestos de conjugación (ácido glucurónico y compuestos de sulfato), producidos en el hígado, parecieron ser la principal vía para neutralizar la toxicidad del DHP. Otro trabajo reveló que *S. jonesii* es un organismo ubicuo que se puede detectar en el líquido ruminal de animales en todos los países, pero siempre en poblaciones bajas, muchas veces solo detectables usando nuevos métodos basados en PCR y a veces no detectadas en todos los animales examinados.

En vista de que el ganado indonesio alimentado con leucaena mostró síntomas de toxicidad por mimosina solo por poco tiempo y se recuperó rápidamente, nuestra hipótesis es que la conjugación del DHP por el hígado es la principal vía de detoxificación en estos animales. Esta vía de detoxificación también se presenta en Australia y otros países pero se necesitan estudios para determinar su significancia.

Palabras clave: Conjugación, detoxificación microbiana, leguminosas arbóreas, 'leucaena bug', rumiantes.

Background

Concern about mimosine toxicity and its management has contributed to the restricted adoption of leucaena as a forage for ruminants. Along with other factors [establishment and management limitations, the psyllid insect (*Heteropsylla cubana*) and weediness concerns (Buck et al. 2019; Dahlanuddin et al. 2019], toxicity concerns have prevented the realization of the huge potential of leucaena pastures as the most productive, sustainable and profitable improved pasture option for northern Australia (Shelton and Dalzell 2007), and for many other tropical regions worldwide (Aung 2019; Chará et al. 2019; Nimbkar 2019; Pachas et al. 2019; Ramírez-Avilés et al. 2019; Zapata Cadavid et al. 2019). The toxicity of leucaena results from the presence of a non-protein free amino acid, mimosine, which occurs in high concentrations in its foliage (Honda and Borthakur 2019) and can severely affect animal health and performance (Jones and Lowry 1984).

The mode of toxicity is initially due to the antimitotic effects of mimosine, which are most pronounced on rapidly dividing cells, causing hair loss, salivation, oesophageal lesions, low bull fertility, foetal abortion and occasionally death (Hegarty et al. 1964; Jones et al. 1978; Holmes 1980; Holmes et al. 1981). However, after an initial adaptation period in ruminants (1–2 weeks), mimosine is rapidly and effectively converted to less acutely, but still toxic compounds, stepwise through isomers of hydroxypyridone (3,4-DHP and then 2,3-DHP). Plant enzymes are involved in the initial conversion of mimosine to 3,4-DHP (Lowry et al. 1983). Thereafter, mimosine does not appear in urine samples (O'Reagain et al. 2014). However, DHP is chronically toxic and was reported to be a goitrogen inhibiting thyroid hormone synthesis, plus reducing feed intake and animal performance (Jones and Lowry 1984). Both compounds have toxic effects as strong ligands and chelate with essential metal ions leading to mineral deficiencies (Tsai and Ling 1971).

R.J. Jones and colleagues conducted the pioneering research into leucaena toxicity between 1960 and 1994 (Hegarty et al. 1964; Allison et al. 1992; Jones 1994) and published widely on the symptoms, chemistry, microbiology and management of toxicity (Jones 1994).

They identified a rumen bacterium (*Synergistes jonesii*) capable of completely degrading DHP in vitro, and rumen fluid containing this organism was subsequently made available as a commercial inoculum for cattle producers in Queensland by Queensland Department of Agriculture and Fisheries (DAF) (Klieve et al. 2002). While this resolved the problem within Australia, an equivalent service was not available in other tropical countries. Despite this, while fear of toxicity has limited the expanded use of the legume in some countries, e.g. Paraguay (Glatzle et al. 2019), many farmers in Asia (Phaikaew et al. 2012) and Latin America (Ramírez-Avilés et al. 2019) have a long history of feeding leucaena to ruminant animals without inoculation, and appear to experience no long-term effects of leucaena toxicity.

Research workers from University of Queensland and CSIRO began studying leucaena toxicity in 2003 and immediately found anomalies and discrepancies with earlier reports, that indicated *S. jonesii* was not as effective as reported (Dalzell et al. 2012; Halliday et al. 2013, 2018) and that there were other mechanisms for neutralizing the toxins in ruminants consuming leucaena (Halliday et al. 2018). The many studies conducted during 2003–2016 are briefly reviewed and a new hypothesis provided to explain how cattle adapt to diets containing high percentages of leucaena. The implications of the hypothesis for future R&D on leucaena toxicity are also discussed.

The evidence

Australia (2003–2011)

Following a report of mortality of hungry cattle when introduced to lush leucaena during a drought in January

2003 (Dr Bevan Peters pers. comm.), a survey of the urine chemistry of a sample of Australian cattle herds grazing leucaena was conducted in 2004 (Dalzell et al. 2012). It showed that half of all herds studied (all grazing leucaena) had high levels of DHP in urine, indicating that it was not being completely degraded despite previous inoculation with rumen fluid by graziers or use of alternative strategies for introducing *S. jonesii* to their herds (Dalzell et al. 2006). Testing for effectiveness of toxin degradation involved detection of the amount of undegraded DHP in urine samples. Jones (1994) employed a simple crush-side colorimetric test in which acidified $FeCl_3$ was added to the urine samples leading to color changes (red color for mimosine and 3,4-DHP and blue color for 2,3-DHP). It was later discovered that incomplete hydrolysis of conjugated DHP was occurring in the colorimetric test, leading to the underestimation of the concentration of DHP in the urine samples (Halliday 2018). A modified colorimetric urine test protocol was developed to provide a more robust and reliable routine test (Graham et al. 2014). This involved collecting and storing urine samples in HCl, and heating them for 1 hour at 80 °C, prior to conducting the $FeCl_3$ colorimetric test and high performance liquid chromatography (HPLC). However, HPLC analysis revealed that incomplete hydrolysis of conjugated DHP continued to occur, resulting in ongoing underestimation of the amount of undegraded DHP in urine samples (Halliday 2018).

Pen-feeding studies found that the commercially available inoculum from DAF was not fully effective in degrading all DHP in steers fed leucaena rations (Halliday et al. 2018). It was originally postulated that, while ruminants were inherently capable of degrading mimosine to the isomer 3,4-DHP via plant and microbial enzymes, the isomerization of 3,4-DHP to 2,3-DHP (Allison et al. 1994) required *S. jonesii*. Therefore, presence of the isomer 2,3-DHP in urine samples was regarded as an indication that bacterial degradation had begun and that 2,3-DHP was transitory. However, several studies showed that this was not the case and that 2,3-DHP was frequently the dominant isomer found in urine samples from cattle on long-term high leucaena diets (Dalzell et al. 2012; Halliday et al. 2014a).

Mexico and Thailand (2005–2009)

A survey of the toxicity status of goat herds in Mexico in 2005 (H.M. Shelton unpublished data) and in Thailand in 2009 (Phaikaew et al. 2012) showed that many herds fed diets of predominantly leucaena (often 100% leucaena diets) were excreting very high levels of DHP. In Thailand, herd averages for total urinary DHP concentrations ranged from 375 to 3,357 µg/mL with most herds excreting >1,000 µg/mL, the majority as 2,3 DHP, indicating that the toxin was not being fully degraded and confirming the Australian findings. Despite this, the goats appeared healthy and productive (Phaikaew et al. 2012).

Indonesia (2011–2016)

The main evidence for an additional pathway of toxin management was discovered in Indonesia. An ACIAR-funded project (LPS/2008/054) (2011–2016) (Shelton 2017) found that, for more than a decade, hundreds of Balinese farmers on the island of Sumbawa had been feeding up to 100% leucaena to Bali bulls (*Bos javanicus*) in profitable fattening enterprises (Panjaitan et al. 2014; Dahlanuddin et al. 2019). Similar practices were observed in West Timor (Kana Hau and Nulik 2019) (Figures 1 and 2).

Figure 1. Bali bulls consuming 100% leucaena diets on Sumbawa Island, Indonesia.

Figure 2. Bali bull fattened on 100% leucaena diet on Sumbawa Island, Indonesia.

The Indonesian animals had not been inoculated with *S. jonesii* and liveweight gains and other measurements showed that they were free from toxicity symptoms and were growing at rates similar to their genetic potential (Panjaitan et al. 2014). When questioned, Indonesian farmers reported that newly purchased cattle, naïve to leucaena, initially showed toxicity symptoms, such as hair loss, salivation and reduced appetite, but recovered within 2–3 weeks and subsequently showed excellent growth performance.

Subsequent tests showed that the cattle were not degrading all DHP as it appeared in high concentrations in urine samples, predominantly as 2,3-DHP (Halliday et al. 2014a; 2014b). However, HPLC analysis revealed almost all (>97%) DHP present in urine was in a conjugated form, detected using a PDA detector (HPLC diode array detector) and by analysis of the UV absorption spectra of the chromatographs (Halliday 2018). The conjugating compounds (glucuronic acid and sulfate compounds) are produced in the liver and are especially effective in conjugating hydroxy compounds, such as DHP (Hegarty et al. 1979). They act by bonding with DHP, neutralizing its toxic activity and increasing its solubility, enabling rapid excretion in urine.

In concurrent microbiological investigations, analysis of rumen fluid from the Indonesian cattle revealed the presence of different strains of *S. jonesii*, including the ATCC type strain (78-1) (Padmanabha et al. 2014; Halliday 2018), albeit at low population levels ($<10^6$ cells/mL rumen fluid), and always accompanied by high levels of DHP in urine. We concluded that *S. jonesii*, while present, was incapable of degrading all the DHP generated from high leucaena diets and that conjugation played a key role in preventing DHP toxicity.

Other evidence

The work of Padmanabha et al. (2014) and McSweeney et al. (2019) showed that *S. jonesii* was not specific to regions where leucaena was being fed, but was an ubiquitous organism detectable in many ruminants, in all countries tested including cold climates (e.g. yaks in Tibet), and in a variety of non-ruminants, but always at low population numbers ($<10^6$ cells/mL rumen fluid) using new PCR-based assays, and sometimes not detectable in all animals studied. They further observed that strains of *S. jonesii* that differed from the type strain 78-1 occurred within animals and at different geographical locations. However, these studies were unable to determine whether there is variation in the DHP-degrading ability of the different strains, which may influence their contribution to the overall detoxification process in the animal. It has also been observed that the main substrates for *S. jonesii* and related genera in the *Synergistetes* phylum are amino acids and their survival does not appear to depend on the presence of DHP, i.e. it is not specifically a 'leucaena bug' as often reported.

In re-examining HPLC chromatographs from earlier studies in Australia (Dalzell et al. 2012; Graham et al. 2013; Halliday 2018), it was evident that additional conjugated DHP was also present in urine samples taken from Australian cattle consuming leucaena. This indicated that the toxic effects of DHP were also reduced by conjugation in Australian cattle consuming leucaena and that the amount of DHP present in those samples had been underestimated. However, since the samples had been immediately acidified at the point of collection, it was not possible to re-estimate the level of conjugated DHP present in the urine.

Discussion, conclusions and future research

We propose that hepatic conjugation was the major pathway for control of DHP toxicity in Indonesian cattle consuming high leucaena diets. Since indigenous strains of *S. jonesii* were already present, albeit at low population density, and almost all excreted DHP was conjugated, presumably negating its toxic effects, and since the animals were gaining weight at a rate close to their genetic capacity, we conclude that inoculation with *S. jonesii* was not necessary in these ruminants.

The process of conjugation of DHP in ruminants has long been recognized, as hydrolysis of the conjugate was a necessary step in the method for measurement of DHP (Hegarty et al. 1964), although it was not historically considered a protective mechanism (Hegarty et al. 1979). The initial focus of DHP toxicity was on its inhibition of thyroid hormones (Jones and Hegarty 1984), even though the conjugated form of DHP had less negative effect on thyroid function than unconjugated DHP (Christie et al. 1979), since conjugation reduces the biological activity of the toxin (Galanello 2007; Crisponi and Remelli 2008). Conjugation also increases the water solubility of the compound, increasing the speed of its clearance in urine (Galanello 2007; Sooriyaarachchi and Gailer 2010).

Contrary to much of the original work on the goitrogenic nature of DHP (Jones et al. 1978; Jones and Hegarty 1984), goitre is rarely observed in leucaena-fed ruminants worldwide. Reduced thyroxin levels were not encountered following the feeding of high leucaena diets to steers in the work of Halliday et al. (2018), suggesting that conjugation of DHP may diminish any direct toxic

activity of the compound on tissues such as the thyroid gland. Conjugation would also reduce the potential for DHP to bind with divalent transition metals such as Zn, Mg and Cu, which are essential for regular cellular function (Berdoukas et al. 1993; Hoffbrand and Wonke 1997). Jones et al. (1978) reported that supplementation of steers on a sole diet of leucaena with minerals (Fe, Cu, Zn) significantly increased mean daily intake and daily liveweight gain, and decreased hair loss and skin lesions although it did not alleviate the low serum T4 levels.

While mimosine can initially induce severe toxicity symptoms such as hair loss, salivation, foetal abortion and even death, it is rapidly and effectively converted to DHP by plant and microbial enzymes. Thus naïve animals, when first fed high leucaena diets, can show symptoms of toxicity, but recover within 2–3 weeks and acute toxicity resulting in death rarely occurs. The Indonesian cattle required a short period of adaptation to firstly degrade mimosine to DHP and then become fully capable of conjugating DHP, thereby preventing negative effects on health and productive performance.

The practical implications of our findings were that feeding of diets containing up to 100% leucaena by Indonesian smallholders was successful in providing a low-cost, low-labor feed source for the productive fattening of bulls (Halliday 2018).

Our current hypothesis after 1.5 decades of research into leucaena toxicity, arising principally from our Indonesian studies, is: when naïve ruminants are introduced to leucaena the mimosine consumed causes immediate symptoms (hair loss, salivation and reduced appetite), from which animals quickly recover as mimosine is converted to DHP. Our understanding of mimosine degradation remains unchanged – plant and microbial enzymes have the capacity to deal effectively with high concentrations of mimosine in the diet within 2–3 weeks in naïve ruminants. Thereafter, conjugation of hydroxypyridone (DHP) plays the major role in protecting animals from residual leucaena toxicity when they consume high leucaena diets. Microbial detoxification by low populations of S. *jonesii*, or by other organisms (Aung 2019), may also play a role but their relative contributions need to be quantified in terms of the amount of DHP degraded.

This finding, if confirmed as applicable to other countries in the tropical world, has great significance for the adoption and use of leucaena for feeding ruminant livestock.

There are several possible explanations for the differences between our current hypothesis and that previously reported, with regard to the need for inoculation with *S. jonesii*, namely:

- There have been advances in methodologies, particularly in rumen molecular techniques, enabling detection of *S. jonesii* when present in the rumen at low populations; and
- We have new understanding of the sample preparation necessary (acid strength and heating requirements) to achieve complete hydrolysis of conjugated DHP prior to measurement by colorimetric or improved HPLC techniques. All previous measurements of concentrations of DHP in urine were almost certainly substantial underestimates.

Future research needs

Confirmation that inoculation of ruminants with *S. jonesii* may not be necessary removes a major world-wide barrier to adoption of leucaena for feeding ruminants. Nevertheless, while there is evidence of similar hepatic conjugation in ruminants consuming leucaena in Australia and other countries where leucaena is being fed, our hypothesis needs to be confirmed by additional studies in those countries.

A number of issues still need clarification, namely:

- More work is required to understand the relative significance of chelation versus effects on thyroid hormones as the principal mode of toxicity of DHP;
- What alternative pathways exist for the isomerization of 3,4-DHP to 2,3-DHP?;
- What rumen organisms can degrade DHP other than *S. jonesii*? An audit of total mimosine ingested and total DHP voided in urine and feces might indicate the possible contribution of other micro-organisms in the detoxification of DHP.
- Are there differences in ability to adapt to leucaena toxicity among species of cattle and between other ruminant species?

Further study is also needed to clarify the effects on the reproductive performance of ruminants of feeding high leucaena diets. Infertility in cattle grazing leucaena was reported by Holmes (1980) and Holmes et al. (1981) in Papua New Guinea. Recent anecdotal evidence (O'Neill and O'Neill 2019) from Australia indicates that foetal abortion can occur when females in the first trimester of pregnancy, naïve to leucaena, are fed high-leucaena diets, leading to lowered calving percentages. In contrast, high calving percentages are achieved in breeding herds where females have adapted to high leucaena diets (J. Schmidt and P. Larsen pers. comm.). Thus, it may

be possible to avoid negative effects on herd reproduction by appropriate herd management.

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ILC2018 Keynote paper*

Detection of *Synergistes jonesii* and genetic variants in ruminants from different geographical locations

Detección de Synergistes jonesii *y variantes genéticas en rumiantes de diferentes regiones geográficas*

CHRIS S. MCSWEENEY[1], JAGADISH PADMANABHA[1], MICHAEL J. HALLIDAY[2], BEN HUBBARD[1,2], LEANNE DIERENS[1], STUART E. DENMAN[1] AND H. MAX SHELTON[2]

[1]*CSIRO Animal Food and Health Sciences, Brisbane, QLD, Australia.* csiro.au/en/Research/AF
[2]*School of Agriculture and Food Sciences, The University of Queensland, Brisbane, QLD, Australia.* agriculture.uq.edu.au

Abstract

Leucaena leucocephala is a nutritionally rich forage tree legume that contains a toxic non-protein amino acid, mimosine, from which other toxic compounds 3,4-dihydroxypyridone (3,4-DHP) and 2,3-DHP are formed in the rumen. The rumen bacterium *Synergistes jonesii* is able to degrade these DHP isomers into non-toxic end products. In this study we developed new PCR-based assays to improve the specificity and sensitivity of detection of *S. jonesii* in the rumen. Using these new assays in a survey of ruminants from different countries, *S. jonesii* appeared to be ubiquitous rather than isolated geographically. The bacterium was present as a minor population (<10^6 cells/mL) in the rumen and was usually comprised of several genetic variants of the species. Although the indigenous nature of *S. jonesii* could imply animals are protected from toxicity, the relative abundance of the bacterium, potential variation in DHP-degrading ability of genetic variants, and amount of leucaena in the diet may determine the ability of the resident population in the rumen to protect the animal from toxicity.

Keywords: Leucaena; rumen fluid; single nucleotide polymorphisms (SNPs); tree legumes; 16S PCR; 2,3 & 3,4-DHP.

Resumen

Leucaena leucocephala es una leguminosa arbórea forrajera rica en nutrientes que contiene mimosina, un aminoácido no proteico tóxico, a partir de la cual se forman otros compuestos tóxicos en el rumen, tales como 3,4-dihidroxipiridona (3,4-DHP) y 2,3-DHP. La bacteria ruminal *Synergistes jonesii* es capaz de degradar estos isómeros de DHP en productos finales no tóxicos. En este estudio desarrollamos nuevos procedimientos basados en PCR para mejorar la especificidad y sensibilidad de la detección de *S. jonesii* en el rumen. Usando estos nuevos procedimientos, un estudio con rumiantes de diferentes países mostró que *S. jonesii* parece ser ubicuo en lugar de aislado geográficamente. La bacteria estuvo presente en una población menor (<10^6 células/mL) en el rumen y generalmente estuvo representada por diferentes variantes genéticas de la especie. Aunque la naturaleza indígena de *S. jonesii* podría implicar que los animales están protegidos de la toxicidad, concluimos que la abundancia relativa de la bacteria, la variación potencial de las variantes genéticas en su capacidad de degradar el DHP, y la cantidad de leucaena en la dieta pueden determinar la capacidad de la población residente en el rumen para proteger al animal de la toxicidad.

Palabras clave: Leguminosas arbóreas; leucaena; líquido ruminal; polimorfismos de nucleótido simple (SNPs); 16S PCR; 2,3 & 3,4-DHP.

Introduction

Leucaena leucocephala is a nutritionally rich forage tree legume that contains a non-protein amino acid, mimosine, which is degraded by ruminal bacteria to the metabolites 3-hydroxy-4(1*H*)-pyridone (3,4-DHP) and 3-hydroxy-2(1*H*)-pyridone (2,3-DHP). Both these isomers of DHP are toxic and can result in impaired thyroid-like symptoms,

Correspondence: C.S. McSweeney, CSIRO Animal Food and Health Sciences, Brisbane, QLD 4072, Australia.
Email: chris.mcsweeney@csiro.au

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chelation of metal ions, reduced liveweight gain and loss of appetite in animals. Raymond Jones discovered a bacterium in the rumen of Hawaiian goats that degraded these DHP metabolites into non-toxic end products (Jones and Lowry 1984), which was later isolated and named *Synergistes jonesii* (Allison et al. 1992). Subsequently, an inoculum containing *S. jonesii* was developed in Australia from a mixed rumen sample taken from a Hawaiian goat and has been used for many years as an 'oral cattle drench' in northern Australia (Jones and Meggarity 1986; Jones 1994; Klieve et al. 2002).

A survey of cattle herds grazing on leucaena in northern Australia showed high levels of 3,4- and 2,3-DHP excretion in urine, despite the majority of herds having been exposed to the 'oral drench' containing *S. jonesii* (Dalzell et al. 2012). The study did not attempt to detect *S. jonesii*, but concluded that a high level of 2,3-DHP (microbial metabolite of 3,4-DHP) in urine indicated its presence, albeit with incomplete degradation of the toxin (Dalzell et al. 2012). However other yet-to-be-discovered species of rumen bacteria, which degrade DHP isomers, could also be present in the rumen. Recently a new hypothesis has emerged which suggests that hepatic conjugation of DHP may contribute significantly to the detoxification of the isomers that have not undergone complete microbial degradation in the rumen (Shelton et al. 2019).

Molecular detection of the *S. jonesii* type strain (78-1, ATCC 49833) was first demonstrated by McSweeney et al. (1993) using radiolabelled (^{32}P) and fluorescent-dye conjugated 16S rRNA targeted oligonucleotide probes. Subsequently, primer sets for PCR-based detection and enumeration of *S. jonesii* targeting genomic regions was developed by Yang et al. (1999) and further assessed for sensitivity by Anderson et al. (2004). However the nature/function of this template DNA in the *S. jonesii* genome was unknown, which raises doubts about the potential specificity of this detection method. Klieve et al. (2002) used a pair of 16S rRNA gene (rDNA) primers consisting of a universal bacterial primer (Primer 357F, Lane 1991) and DHP 1006 primer (McSweeney et al. 1993) to amplify a 438 nucleotide product specific for *S. jonesii*. Another set of 16S rDNA primers for *S. jonesii* (sng796f and sng1001r) were used by Derakhshani et al. (2015) but they did not report on their validation. In an attempt to increase sensitivity and specificity of detection, Graham et al. (2013) used a 16S rDNA nested PCR approach to monitor the presence of *S. jonesii* in cattle from northern Australian properties. This method detected *S. jonesii* in <10% of the cattle tested, even though several herds had been inoculated with the bacterium and DHP degradation was occurring. Sequence analysis of the 16S rDNA amplicons from samples positive for *S. jonesii* showed that all had differing sequence profiles compared with the *S. jonesii* ATCC type strain 78-1. Another survey of ruminants in different geographical regions confirmed the presence of *S. jonesii* in cattle, goats, sheep, yak and buffalo from Australia, China, Brazil, Thailand, Indonesia and Vietnam by an improved nested 16S rDNA PCR approach (Padmanabha et al. 2014). Sequence analysis of these PCR products revealed at least 4 loci with point mutations (single nucleotide polymorphisms; SNPs) compared with the ATCC type strain. The specificity of the PCR assay was further improved by Halliday et al. (2018) but showed <50% of rumen samples were positive for *S. jonesii* in a group of cattle partially degrading DHP. These studies indicate the bacterium is often present but below the limit of detection (10^4–10^5 cells/mL) and therefore improved molecular assays are required for monitoring populations of *S. jonesii* in vivo.

The present study reports on further improvements in sensitivity of the PCR method for detecting the presence of *S. jonesii* in the rumen by using cDNA generated from rRNA as template for the PCR assay rather than its genomic DNA (gDNA). New primer sets were designed to amplify SNP regions of the 16S rDNA and these primers were also used in nested PCR, quantitative PCR (qPCR) and reverse transcriptase qPCR (RT-qPCR). This study also examined the geographical distribution relating to variations in the 16S rDNA of the *S. jonesii* strain 78-1 which may suggest divergence from the type strain in Australian cattle as well as in ruminants internationally. These changes may be correlated with the ability of the bacterium to degrade DHP, relative to the type strain.

Materials and Methods

Rumen fluid collection

Rumen fluid (RF) was collected mainly by orogastric tube from Australian cattle and from cattle, sheep, goats, buffalo, native cattle and yak from Indonesia, Thailand, Vietnam, China, Scotland and Brazil as well as gut samples or feces from some non-ruminant herbivores (see Table 2). Australian samples were generally stored on ice before freezing at -80 ºC. Rumen samples from overseas were preserved in 100% ethanol for transportation at room-temperature prior to DNA extraction. Some rumen samples were also preserved for RNA extraction by mixing rumen fluid with an equal volume of RNA*Later*™ Stabilization Solution (Thermo Fisher Scientific, Australia). These samples were chilled at ~2–4 ºC for 24–48 h, centrifuged at 6,000 × g for 15 minutes and the pellet alone re-suspended in 70% ethanol for storage and transport at ambient temperature. Samples were stored at -80 ºC until DNA or RNA extraction.

DNA extraction

Genomic DNA was extracted from 2 mL culture/rumen fluid pellets/feces, harvested by centrifugation for 20 min at 17,000 × g at 4 ºC, using the cetyltrimethylammonium bromide (CTAB) method of Jones and Walker (1963) and Murray and Thompson (1980) with a modified bead-beating method (Gagen et al. 2010). Briefly, cell pellet from 2 mL rumen fluid sample was re-suspended in 800 µL of CTAB isolation buffer (100 mM Tris-HCl, pH 8; 1.4 M NaCl; 20 mM EDTA-disodium salt; 2% CTAB) and homogenized in a FastPrep®-24 bead-beater with zirconium-silica beads before extracting the DNA, quantifying and storing at -20 ºC until used for PCR/qPCR analyses as described by Halliday et al. (2018). The gDNA from rumen digesta samples was diluted approximately to 50–100 ng/µL and used in a 25 µL PCR or qPCR.

RNA extraction and cDNA synthesis

Total RNA was extracted from samples either stored in RNA*Later*™ or RNA*Later*™ followed by ethanol preservation. *Synergistes jonesii* pure culture cells harvested as standards for Reverse-Transcriptase qPCR (RT-qPCR) were initially 'fixed' by adding 1:1 volume of 5% phenol: ethanol (v/v), pH 4.3, mixed at 4 ºC to prevent RNA degradation and the pellets stored frozen (-80 ºC). A pellet from a 1 mL sample of culture or rumen fluid was harvested by centrifugation at 17,000 × g, 4 ºC for 10 min and was re-suspended in (in order): 300 µL 10 × TE buffer pH 6.0 (Tris-EDTA buffer); 400 µL phenol:chloroform (1:1), pH 4.3, (Sigma-Aldrich, USA); and 100 µL 10% sodium dodecyl sulphate (SDS). Further, UV-sterilized 250 mg of zirconia/silica beads (0.1 mm and 1 mm, 1:1 w/w) was added to each tube and homogenized in a FastPrep-24® bead-beater (MP Biomedicals, USA) for 3 × 1 min at setting 6.5, with a rest of 1 min between cycles. The homogenized suspension was centrifuged as before and the aqueous phase transferred to a new tube for silica-gel column purification. The RNA was extracted using RNeasy® Mini Kit (QIAGEN GmbH, Germany) following manufacturer's protocol with these modifications: 500 µl RLT buffer was added to the aqueous phase, mixed well, followed by adding 500 µL 96% ethanol to precipitate the RNA. This mixture was applied to an RNeasy Mini spin column, in 2 lots of 700 µL each and centrifuged at 8,000 × g for 15 sec to bind the RNA to the silica column and washed with RW1 wash buffer. Residual DNA contamination of the RNA was removed by on-column digestion using manufacturer's DNase mix in RDD buffer and procedure, washed with RW1 wash buffer and twice with RPE buffer. The column was dried by a final centrifugation for 2 min at 10,000 × g and RNA eluted in 50 µL RNase free water and mixed with 1 µL RNaseOUT™ RNase Inhibitor (Thermo Fisher Scientific, Australia). The rRNA was quantified and RNA Integrity (RIN) assessed on an Agilent 2100 Bioanalyzer using Agilent RNA 6000 Nano chip Kit (Agilent Technologies, Santa Clara, CA) following the manufacturer's instructions for prokaryotes. Total RNA was frozen at -80 ºC.

Total RNA was converted to cDNA using the Superscript® III Reverse Transcriptase (Thermo Fisher Scientific, Australia) using 2 pmol of *S. jonesii*-specific primer (Sj_1004R: 5'-CCT CTC GAT CTC TCT CAA GTA AC-3') following manufacturer's protocol. Briefly, 1 µL of 2 µM *S. jonesii*-specific primer, 1 µL 10 mM dNTP mix and ~100 ng total RNA (typically 2 µL) was brought to a final volume of 14 µL and denatured at 65 ºC for 5 min in a PCR machine and placed on ice for 3 min. To this, a master mix containing 4 µL, 5 × First-Strand buffer; 1 µL, 0.1 M DTT; and 1 µL, Superscript III RT (200 units/µL) was added and cycled in a PCR at 25 ºC for 5 min; 50 ºC for 60 min; and 70 ºC for 15 min. The cDNA was frozen (-80 ºC) or stored at 4 ºC for analysis.

Detection of Synergistes jonesii *by nested PCR and RT-qPCR*

A nested PCR approach based on the 16S rDNA was used to detect the presence of *S. jonesii* using specific primer sets including new primers described by Halliday et al. (2018) (Table 1). The *S. jonesii*-specific cDNA generated from 16S rRNA was used as a template for RT-qPCR with gene-specific primers, Sj_60F and Sj_449R (Table 1), using SensiFAST™ SYBR® Lo-ROX Kit (Bioline Reagents Ltd, UK) following the manufacturer's procedure. Each qPCR reaction was performed in quadruplicate (10 µL each) from a master mix with (1 µL/25 µL) cDNA template and aliquoted into MicroAmp® Optical 384-well reaction plate (Applied Biosystems, Life Technologies). The qPCR was run on Applied Biosystems ViiA™ 7 Real-Time PCR system with the following parameters: 95 ºC for 3 min; 40 cycles of 95 ºC for 15 sec and 60 ºC for 1 min; and a final melt curve analysis consisting of 95 ºC for 15 sec, 60 ºC for 60 sec and 95 ºC for 15 sec. Analysis was completed using QuantStudio™ Real-Time PCR software (Applied Biosystems, Carlsbad, CA). The sensitivity of detection of the nested PCR using gDNA compared with rRNA (converted to cDNA) was assessed by serial dilutions of

Table 1. Primer sets used in this study.

Primer Name	Primer Sequence (5'-3')[1]	Used for	Reference
SJ_60F	AGT CGA ACG GGG ATC ATG T	Nested PCR, qPCR, Sequencing	Halliday et al. 2018
SJ_1039R	CCA TGC AGC ACC TGT TCT AC	Nested PCR	Halliday et al. 2018
SJ_1004R	CCT CTC GAT CTC TCT CAA GTA AC	cDNA synthesis, Nested PCR, Sequencing	Halliday et al. 2018
SJ_449R	CGT CAC TCG CTT CTT CCC GC	qPCR	This study
SJ_60F-Linker	**CGA TTC ATT AAA GCA GAT CTC GAT CCC** AGT CGA ACG GGG ATC ATG T	Pyrosequencing	This study
SJ_449R-454B	CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG *CAA CAG CT* CGT CAC TCG CTT CTT CCC GC	Pyrosequencing	This study
AbcL	CCATCTCATCCCTGCGTGTCTCCGACTCAG-bc-**CGATTCATTAAAGCAGATCTCGATCCC**	Pyrosequencing	Aguirre de Cárcer et al. 2011

[1]Bold = linker sequence; Dotted underline = 454-adaptor B; Double underline = 454 adaptor A; -bc- = unique 8 bp barcode; Underline = target specific sequence; Italics = padding sequence.

mixed rumen culture seeded with *S. jonesii* 78-1 cells as described previously (Halliday et al. 2018). Amplicons from *S. jonesii* nested PCR-positive samples were Sanger sequenced to confirm identity as described in Halliday et al. (2018).

Survey S. jonesii *SNPs using high-throughput sequencing*

A survey of livestock from different countries for *S. jonesii* SNP distribution was done by 16S rDNA-based methods using a nested 454 pyrosequencing (454)-barcoding PCR as described by Aguirre de Cárcer et al. (2011) with minor modifications. Firstly, a primary PCR using *S. jonesii*-specific primer pair SJ_60F-SJ_1004R (Table 1) for 25 cycles was run. An aliquot (~5 µL) of each of this PCR product was cleaned using ExoI/CIAP (37 ºC for 20 min and 80 ºC for 20 min) followed by a 20 cycle nested PCR using primer pair SJ_60F-Linker-SJ_449-454B (Table 1) to generate *S. jonesii* 16S rDNA products (~380 bp V2/V3 region) for the SNP region. Finally, a 454-barcoding PCR run was completed on the cleaned 15–20 µL aliquot of the positive nested-PCR products using unique 8 bp error-correcting barcodes with the 454 adaptor A and SJ_449R_454B primer pair (Table 1) for 10 cycles. The barcoded products were quantified using the Quant-IT dsDNA Assay kit (Thermo Fischer Scientific, Australia), pooled at equimolar amounts, concentrated, electrophoresed and gelpurified (QIAquick Gel Extraction Kit, QIAGEN GmbH, Germany). The final purified product was sequenced using a Roche 454 GS-FLX Titanium sequencer at Macrogen Inc. (Seoul, Korea).

Data analysis

Short read sequence data generated using 454 pyrosequencing for *S. jonesii* SNP patterns was analyzed using QIIME (Quantitative Insights Into Microbial Ecology) software package (Caporaso et al. 2010). Briefly, the sequences were demultiplexed based on barcode sequences and filtered for a minimal average quality score of 25 across a 50 bp sliding window and trimmed for length ranging from 300 to 600 bp. Raw sequences were passed through Acacia for 454-error correcting (Bragg et al. 2012). Error-corrected sequences were then clustered to OTUs at 100% similarity for *S. jonesii* performed using UCLUST algorithm (Edgar 2010). Chimeric sequences were identified using Chimera Slayer (Hess et al. 2011) and removed. Representative sequences were aligned to the Green Genes reference database (McDonald et al. 2012) using the RDP classifier software (Wang et al. 2007; Werner et al. 2012). Additional analysis of OTUs was performed in the R packages ade4 and Phyloseq (Chessel et al. 2004; McMurdie and Holmes 2013) and identification of the SNP pattern was achieved by filtering the alignment with a *S. jonesii*-specific lane mask for the 3 SNP-base positions.

Results

Detection of S. jonesii *by nested PCR*

A two-step nested PCR approach was used to detect the presence of *S. jonesii* in gDNA extracted from gut contents of livestock in different countries. The primer pairs SJ_60F-SJ_1039R or SJ_60F-SJ_1004R were used in the primary PCR followed by SJ_60F-SJ_1004R or SJ_60F-SJ_449R primer sets (Table 1). Nearly all groups of animals tested from Australia, Indonesia, Thailand, Vietnam, Scotland, China and Brazil were positive for *S. jonesii* but rarely did all animals in a group return a positive result and <50% of the total tested positive (Table 2).

Table 2. Detection of *S. jonesii* in livestock from different countries using a nested PCR assay.

Country, location	Animal species	Animals (n)	Sample source	Diet	*S. jonesii* positive (n)	Index no. in Figures 2 & 3[#]
AUSTRALIA						
Southeast Queensland						
Various locations	Cattle	4	RF	Grass pasture	4	1
	Cattle	4	RF	Leucaena + Grass pasture	0	-
	Camel	3	Feces	Pasture	1	19
	Lamb	8	RF	Grass pasture	1	16
	Horse	10	Feces	Grass pasture	2	20
Mt Cotton-Period 1	Cattle	16	RF	Lucerne + Leucaena	3	1[+]
Mt Cotton-Period 2	Cattle	16	RF	Leucaena	7	1[+]
Murgon	Cattle	2	RF	Leucaena	2	1*
Central Queensland						
Thangool	Cattle	3	RF	Leucaena	3	1
Belmont Station	Cattle	69	RF	Leucaena-Grass pasture	24	1
North Queensland						
Lansdown Station	Cattle	17	RF	Grass pasture	11	1
Lansdown/Ayr/Mt Garnet	Cattle	32	RF	Grass pasture + Leucaena	20	1*
Northern Territory						
	Cattle	2	RF	Grass pasture	0	-
Victoria						
	Dairy cows	5	RF	Grass pasture	4	2
Western Australia						
	Sheep	8	RF	Grass hay	0	-
INDONESIA						
Sumlili	Goats	3	RF	Leucaena	2	14b
Lombok	Goats	11	RF	Leucaena	11	14a*
Timor	Local cattle	7	RF	Leucaena	1	6*
Jati-Sari	Local cattle	17	RF	Leucaena	1	6
Sumbawa	Local cattle	10	RF	Leucaena	5	7
Sumba	Buffalo	2	RF	Leucaena	2	11
Melolo	Buffalo	3	RF	Leucaena	3	12*
THAILAND						
Khon Kaen Uni.	Buffalo	4	RF	Leucaena	4	13*
	Native cattle	10	RF	Leucaena	10	8*
VIETNAM						
Can Tho Uni. farm	Cattle	6	RF	Grass pasture	1	9*
	Goats	12	RF	Forage + Leucaena	4	15*
CHINA						
Lanzhou Uni. farm	Yaks	19	RF	Alpine pasture	4	10
	Jinnan cattle	7	RF	Alpine pasture + Barley straw	4	5
	Gansu sheep	3	RF	Alpine pasture + Oaten hay	3	18
	Tibetan sheep	3	RF	Alpine pasture + Oaten hay	2	18*
SCOTLAND						
	Cattle	2	RF	Pasture	2	4
	Sheep	2	RF	Pasture	2	17
BRAZIL						
São Paulo Uni. farm	Cattle	10	RF	Grass pasture	6	3

[#] Index number corresponds to sample number in parenthesis used in Figures 2 and 3 for SNP analysis.
S. jonesii positive rumen samples from Padmanabha et al. 2014* and Halliday et al. 2018[+]. RF= rumen fluid.

Detection of Synergistes jonesii *by RT-qPCR*

The RNA-based RT-qPCR increased the sensitivity for detection of *S. jonesii* by more than 100-fold compared with gDNA analysis in mixed cultures of rumen bacteria containing the target bacterium (Figure 1). The majority of rumen samples from 2 groups of cattle in Indonesia and 1 group in Australia, which tested negative for *S. jonesii* based on gDNA analysis (nested PCR), were mainly positive when RNA-based analysis was used (Table 3). This confirmed that the populations of *S. jonesii* were often at the limits of detection for nested PCR.

Survey of SNP diversity in S. jonesii *16S rDNA*

Synergistes jonesii type strain 78-1 contains the SNP variant CAG within the 16S rDNA. The Queensland Department of Agriculture and Fisheries (QDAF) inoculum, that is provided to cattle producers to protect their herds from toxicity, contains a CAG variant as a minor member while the dominant variant (98%) contains the CGG SNP pattern (Figure 2). The majority of livestock samples contained 3 or more of the SNP variants irrespective of their country of origin, while the remaining samples had 2 variants. Samples which did not contain the type strain (78-1) SNP included: Australian dairy cattle; camels and horses; some Chinese cattle and yaks; some Indonesian cattle and goats; and Vietnamese cattle.

When Australian cattle were compared, the most common SNP pattern across animals was CGG, which is also the dominant SNP in the QDAF inoculum (Figure 3).

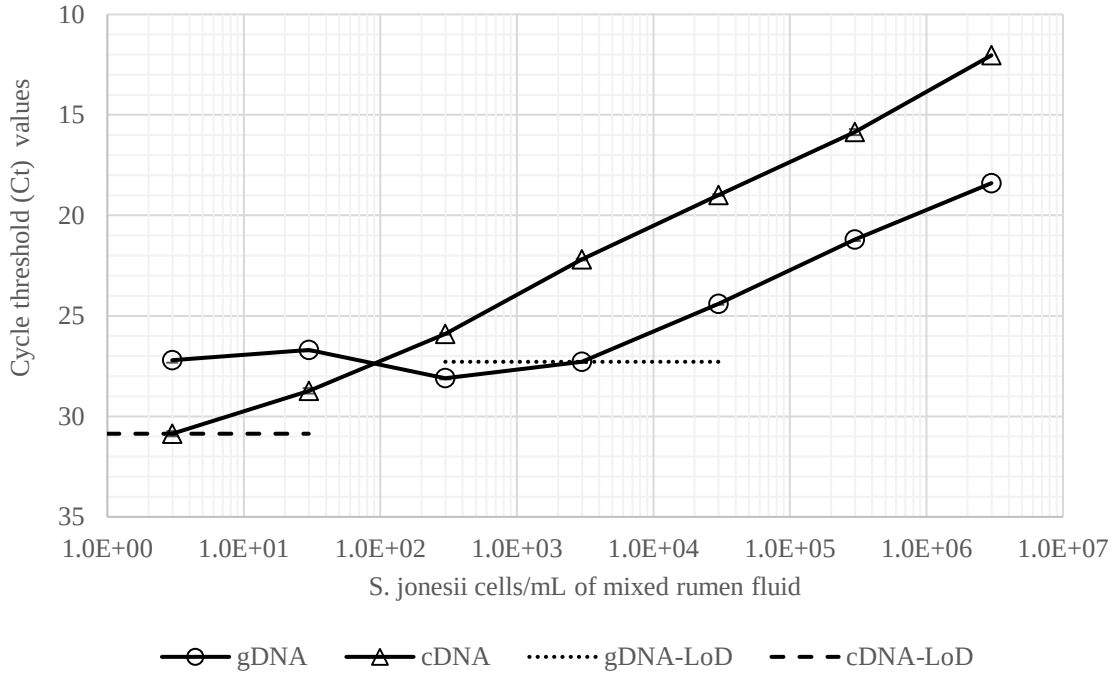

Figure 1. Detection and quantitation of *S. jonesii* cells spiked into mixed rumen culture by qPCR (using gDNA) and RT-qPCR (using cDNA). LoD = limit of detection.

Table 3. Detection of *S. jonesii* in cattle from different regions using gDNA (nested PCR)- and RNA (RT-qPCR)-based assays.

Country, location	Animal	Animals (n)	*S. jonesii* positive (n)	
			RT-qPCR	Nested PCR
Indonesia				
Jati-Sari	Indonesian cattle	34	26	1
Sumbawa	Indonesian cattle	10	10	5
Australia				
S.E. Queensland[1]	Local cattle	16	13	3

[1]Rumen samples from penned-cattle fed different levels of leucaena and administered *S. jonesii* containing inoculum (Halliday et al. 2018).

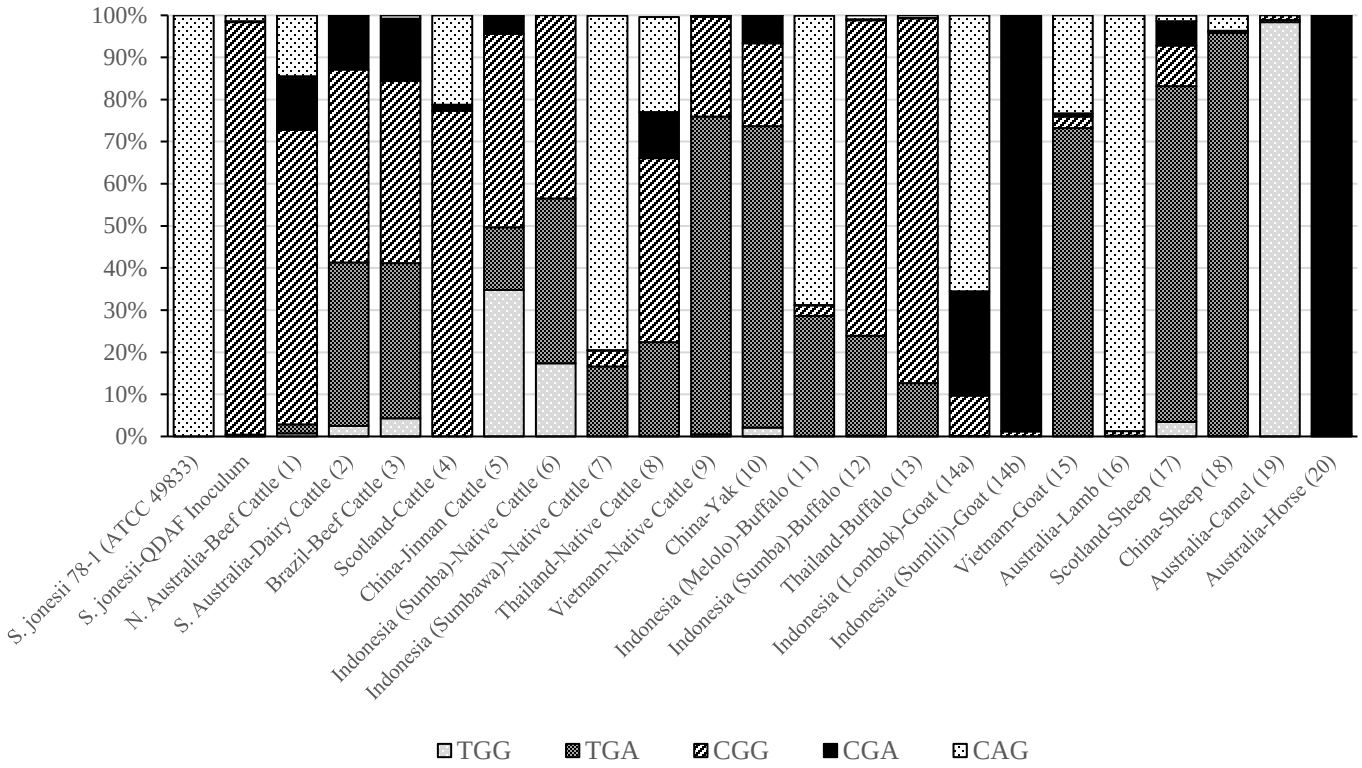

Figure 2. Distribution of *S. jonesii* 16S rDNA-specific SNPs in livestock species from different countries. The index numbers in parenthesis identify the samples from Table 2 used in the SNP analysis.

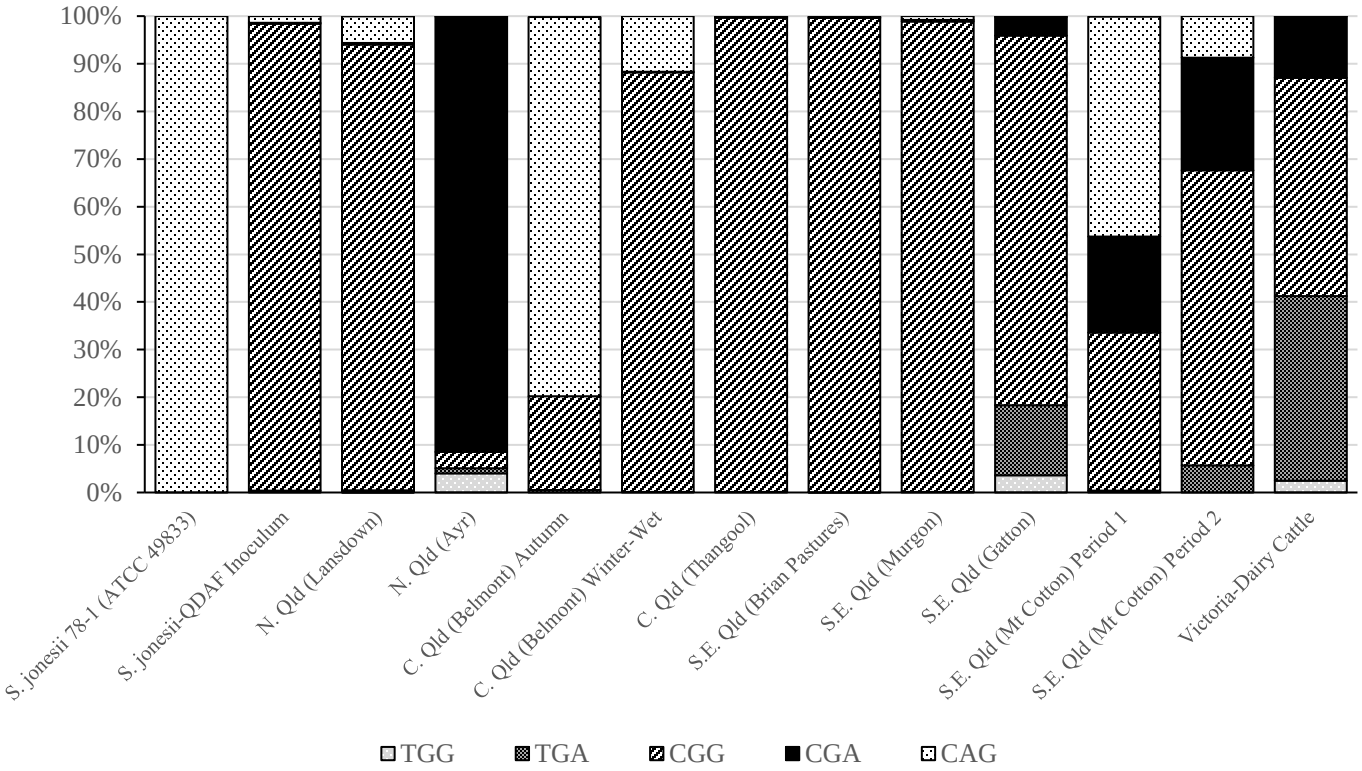

Figure 3. Distribution of *S. jonesii* 16S rDNA-specific SNPs in beef cattle from northern Australia and dairy cattle from Victoria. The SNPs were generated from *S. jonesii* positive samples corresponding to the named sites with index number 1 and 2 indicated in Table 2.

Discussion

The results of this study support the recent discovery that *S. jonesii* appears to be ubiquitous in ruminants rather than isolated geographically (Padmanabha et al. 2014). The nested PCR was able to detect *S. jonesii* usually at low numbers (<10^6 cells/mL) in most of the Australian cattle groups and overseas ruminants (cattle, buffalo, goats, sheep and yaks), whether feeding on leucaena or not, suggesting that the bacterium is indigenous to many of these animals and regions. Clearly, the RNA-based RT-qPCR method increased the sensitivity of detection of *S. jonesii* and would provide a more accurate measure of the prevalence of the bacterium in future surveys provided the rumen digesta-RNA can be immediately preserved upon collection using stabilizing chemicals/agents. Despite the increase in sensitivity, RNA-based PCR is not suitable for quantification of *S. jonesii* as it is based on an unknown number of target 16S rRNA gene copies per bacterium that can vary significantly depending on the stage and rate of growth of the bacterium. However a qualitative assessment of relative abundance could be achieved by performing a dilution series of the rumen fluid and noting the greatest dilution at which a positive test was observed. Increasing the abundance of *S. jonesii* in the rumen by providing peptides or specific amino acids which are required by the bacterium (Allison et al. 1992) has not been studied but should be examined in future as a way of increasing DHP degradation.

Although the indigenous nature of *S. jonesii* would imply inoculation is not required to transfer the microbe, the lack of complete degradation of DHP observed in many animals globally suggests *S. jonesii* alone is incapable of fully protecting ruminants on high leucaena diets. A recent review of leucaena toxicity in ruminants concluded that hepatic conjugation of DHP plays a major role in protecting animals from toxicity of DHP isomers that have not been completely degraded to non-toxic products in the rumen (Shelton et al. 2019). Although *S. jonesii* appears to be ubiquitous, work prior to the development of sensitive and specific molecular PCR assays reported that DHP degradation by rumen microbiota (presumably *S. jonesii*) did not occur in all countries tested (Jones 1984). Collectively these observations indicate that, while *S. jonesii* may be distributed widely in ruminants, their ability to degrade DHP could vary between animals and geographical regions.

The nested PCR amplicons from rumen samples identified as positive for *S. jonesii* were 454-sequenced to determine any variations of the 16S rDNA. Previously, longer (~800 bp) Sanger-sequencing analysis of the *S. jonesii* positive 16S rDNA sequences revealed 4 loci with point mutations at base positions (based on *E. coli* 16S rDNA numbering) 268 (C/T), 306 (A/G), 328 (G/A) and 870 (A/C) (Padmanabha et al. 2014). These are single nucleotide polymorphisms or SNPs, and, when present, occurred predominantly at loci 306 and 870 (Padmanabha et al. 2014). To facilitate a deeper analysis of the frequency of these SNPs in individual rumen samples, we designed PCR primers that would amplify a region that included only the first 3 of the 4 loci (~37–380 bp), as a larger amplicon that included the locus at base 870 would not be amenable to 454-pyrosequence analysis. The SNP analysis demonstrated that the *S. jonesii* population in livestock is usually composed of several strains and never represented solely by the type strain (78-1) SNP (CAG).

The presence of SNPs within the 16S rDNA of the *S. jonesii* species implies there is genetic variation between closely related strains. This strain diversity may account for some of the differences observed in degradation of the 2 DHP isomers between animals. The total amount of undegraded DHP in the urine is most likely correlated with the amount of leucaena in the diet. It is possible that the DHP-degrading ability of different strains may vary, particularly if some *S. jonesii* bacteria are present in ruminants where leucaena and DHP are absent from the environment. Previous studies have demonstrated that the enzymes involved in the isomerization of 3,4-DHP to 2,3-DHP and cleavage of the pyridine ring are regulated by the concentration of pyridinediols (Rincón et al. 2000). The genetic regulation of these metabolic processes may have evolved differently depending on the rumen environment in which the bacteria reside. In a recent study, where cattle were dosed with the QDAF enrichment inoculum, Halliday et al. (2018) provided some evidence that there are differences in the DHP-degradation pathway between strains of these bacteria. They concluded that there were indigenous strains of *S. jonesii* in cattle, which converted 3,4-DHP to 2,3-DHP at a faster rate than 2,3-DHP was able to be degraded. Following inoculation, the extent of 2,3-DHP degradation appeared to increase and total DHP excretion decreased, indicating that the inoculum may have been more effective in degrading 2,3-DHP than the indigenous strains. Therefore despite already harboring indigenous *S. jonesii*, the provision of the QDAF inoculum may further enhance the ability of the rumen to degrade DHP. However it is also possible that other species of DHP-degrading bacteria, which contribute to these differences in metabolism of the two DHP isomers, could be present in the rumen (Allison et al. 1990; Domínguez-Bello et al. 1997).

In conclusion, *S. jonesii* was present as a minor population (<10^6 cells/mL) in the rumen of most animals tested and was usually comprised of several genetic variants of the species. Although the indigenous nature of *S. jonesii* could imply animals are protected from toxicity, the relative abundance of the bacterium, potential variation in DHP-

degrading ability of genetic variants of the species, and level of intake of leucaena may all influence the capacity of the resident population in the rumen to protect the animal from toxicity.

Acknowledgments

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ILC2018 Keynote paper*

Mimosine concentration in *Leucaena leucocephala* under various environmental conditions

Concentración de mimosina en Leucaena leucocephala *bajo diferentes condiciones ambientales*

MICHAEL D.H. HONDA AND DULAL BORTHAKUR

Department of Molecular Biosciences and Bioengineering, University of Hawaii at Manoa, Honolulu, HI, USA.
manoa.hawaii.edu

Abstract

Leucaena leucocephala (leucaena) is a multipurpose tropical tree-legume that is highly resistant to many biotic and abiotic stresses. Leucaena is used primarily as an animal fodder owing to its protein-rich foliage. However, leucaena foliage also contains mimosine, a toxic non-protein amino acid that can cause alopecia, goiter and other thyroid problems, infertility and fetal death. Considering its toxicity and abundance in leucaena, it is important to quantify the mimosine concentrations in leucaena under different environmental conditions. Mimosine was extracted from various types of leucaena tissue exposed to a range of environmental conditions and then quantified by HPLC. The mimosine concentrations in leucaena treated with NaCl increased after 6 days of treatment and remained relatively high when treatment continued for 18 days. Interestingly, leucaena exposed to complete darkness for up to 5 days had a higher mimosine concentration than control plants exposed to normal light/dark photoperiods. On the other hand, drying leucaena leaflets or macerating them in an alkaline buffer significantly lowered their mimosine concentration. Mature leaflets that had fallen off the plant and dried out also contained significantly less mimosine than fresh leaflets. The results of this study indicate that mimosine concentrations in leucaena are affected by environmental conditions and this knowledge can assist in managing to prevent toxicity.

Keywords: Foliage, non-protein amino acid, toxins, tree legumes.

Resumen

Leucaena leucocephala (leucaena) es una leguminosa tropical multipropósito altamente resistente a muchos estreses bióticos y abióticos. Leucaena es usado principalmente para alimentación animal debido a que su follaje es rico en proteínas. Sin embargo, el follaje de leucaena también contiene mimosina, un aminoácido no proteico tóxico que puede causar alopecia, bocio y otros problemas de tiroides, infertilidad, y muerte fetal. Teniendo en cuenta la toxicidad de la mimosina y su abundancia en leucaena, es importante cuantificar sus concentraciones en diferentes condiciones ambientales. En el estudio se extrajo la mimosina de varios tipos de tejido de leucaena, expuesto a una variedad de condiciones ambientales, y se cuantificó por HPLC. Las concentraciones de mimosina en leucaena tratada con NaCl aumentaron después de seis días de tratamiento y se mantuvieron relativamente altas cuando el tratamiento continuó durante 18 días. Sorpresivamente, leucaena expuesta a oscuridad completa durante hasta cinco días mostró una concentración de mimosina más alta que plantas testigo expuestas a fotoperíodos normales de luz/oscuridad. Por otro lado, el secado de los folíolos de leucaena o macerarlos en un buffer alcalino redujo significativamente la concentración de mimosina. Folíolos maduros, desprendidos de la planta y secos, también contuvieron significativamente menos mimosina que folíolos frescos. Los resultados de este estudio indican que las concentraciones de mimosina en leucaena se ven afectadas por condiciones ambientales. Este conocimiento puede ayudar a desarrollar estrategias de prevención de toxicidad.

Palabras clave: Aminoácidos no-proteicos, follaje, leguminosas arbóreas, toxinas.

Correspondence: Dulal Borthakur, Department of Molecular Biosciences and Bioengineering, University of Hawaii at Manoa, Honolulu, HI 96822. Email: dulal@hawaii.edu

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Introduction

Leucaena leucocephala (leucaena) is a fast-growing tree-legume native to Southern Mexico and Central America (Brewbaker 1987). Leucaena is grown in the tropical and subtropical regions of the world for its multipurpose uses, which include fodder for farm animals and pulp for paper production. As a fodder, leucaena is highly palatable and rich in many micro- and macro-nutrients, including iron, fiber and protein (Brewbaker 2016). However, leucaena foliage can also contain high amounts of mimosine, a toxic non-protein amino acid, which is found in all plant parts, including foliage, flowers, seeds, stems, roots and root nodules (Soedarjo and Borthakur 1996). Mimosine toxicity is attributed to its ability to form a stable complex with pyridoxal-5' phosphate (PLP) and metallic ions such as Fe^{3+}, Cu^{2+} and Zn^{2+}. These metallic ions and PLP are important enzyme co-factors for many biochemical pathways. Side-effects of mimosine toxicity resulting from disruption of these pathways include alopecia, infertility, fetal defects and goiter and other thyroid problems (Crounse et al. 1962; Hamilton et al. 1968; Joshi 1968; Dewreede and Wayman 1970). Mimosine is degraded to 3-hydroxy-4-pyridone (3H4P) by mimosine-degrading enzymes, mimosinase and rhizomimosinase, which are present in leucaena and *Rhizobium* sp. strain TAL1145, respectively (Negi et al 2013; 2014). Mimosine is also converted to 3H4P and 2,3-dihydroxypyridine (2,3-DHP) by the microflora present in ruminants (Dominguez-Bello and Stewart 1990). Both 3H4P and 2,3-DHP are also toxic but can be degraded to harmless products by the ruminal bacterium *Synergistes jonesii* (Jones and Megarrity 1986).

In spite of containing mimosine, leucaena is an ideal fodder due to the high protein concentration in its foliage, high fodder yield and resistance to many biotic and abiotic stresses, which include diseases, pests and drought (Shelton and Brewbaker 1994; Honda et al 2018). It is hypothesized that the changes in the mimosine concentration in leucaena are a response to environmental stresses. Therefore, considering its toxicity and possible role in stress resistance, it is important to study the fluctuation of mimosine concentrations in leucaena exposed to a range of environmental conditions.

Materials and Methods

Mimosine and 3-hydroxy-4-pyridone (3H4P) extraction and quantification

To extract mimosine and 3H4P from the various leucaena parts and tissues, 1 g samples of respective plant matter were placed in a 50 mL conical tube. The samples were then submerged in 30 mL of 0.1N HCl and incubated overnight at room temperature while shaking. Previous experience showed that heat and grinding treatments were unnecessary and less efficient for calculating the % dry weight of mimosine, so they were not used in the present study. After overnight incubation, leaflet extracts were spun for 15 min at 12,000 rpm to remove plant debris. The supernatants of leaflet extracts were assayed by HPLC using a Waters 2695 separations module, a Phenomenex C18 column (5µ; 4.6 × 250 mm), and a UV detection photodiode array (280 nm). An isocratic carrier solvent of 0.02 M o-phosphoric acid at a linear flow rate of 1 mL/min was used for HPLC analysis. The leaflet material was rinsed several times with dH_2O then dried in a baking oven. For quantitative determination of mimosine and 3H4P, synthetic mimosine and 3H4P were prepared in various concentrations and then assayed by HPLC following the above methods. The areas under the curves for mimosine and 3H4P peaks were used to plot a standard curve, which was then used to quantify mimosine and 3H4P concentrations in leaflet extracts.

Mimosine in adult leucaena leaflets and shoot tips

Leucaena shoot tips, fresh leaflets and leaflets that had fallen from the plant and dried out, were collected, then separated based on color, size and health of the leaflets. Some fallen leaflets had a reddish color (due to loss of chlorophyll, oxidation and/or increase in pigments such as anthocyanins). Mimosine and 3H4P were extracted from the leaflets, then quantified by HPLC. In another experiment, fresh green leaflets were dried overnight in an oven before mimosine was extracted and quantified by HPLC. Experimental sets were performed in triplicate.

Mimosine in adult leucaena stems

Stems that were no more than 6 mm in diameter from adult leucaena plants (common, i.e. shrubby variety) were harvested, then cut and separated into 3 parts, top, middle and lower sections. Mimosine was extracted from the various stem sections then quantified by HPLC. Each sample set contained 3 biological replicates.

Germination and growth of leucaena seedlings

Mature seeds of leucaena were collected from plants at the University of Hawaii Waimanalo Research Station, Waimanalo, Hawaii. Samples of seeds were submerged in concentrated sulfuric acid and gently agitated at room temperature for 6 min. After scarification, the seeds were rinsed with deionized water then placed in 51 × 25 cm trays containing a vermiculite-soil mixture. The seeds were

allowed to germinate and then grew for 1 month at 25 ± 2 °C with a 16/8 h light/dark photoperiod with an irradiance of 74 µmol/s/m. Plants were watered once a week with quarter-strength Hoagland solution. After 1 month of growth seedlings were transferred to pots (4 per pot) containing a soil-vermiculite mixture. The seedlings were then grown for additional respective times following the methods described above. All treatments were carried out in these pots unless otherwise stated.

Treatment with NaCl

Quarter-strength Hoagland solution containing 300 mM NaCl was applied every 2 days to the growth media of 4-month-old leucaena seedlings. Mimosine was extracted and quantified from the leaflets of leucaena seedlings at 0, 3, 6, 9, 12, 15 and 18 days after initial application of NaCl treatment. Each sample set contained a minimum of 6 biological replicates.

Treatment with various metallic salts

Two-month-old leucaena seedlings were fed every 2 days with quarter-strength Hoagland solution containing water (control), 10 mM $FeCl_3$, 10 mM $ZnSO_4$ or 10 mM $CaCl_2$. After 1 week of treatment, mimosine was extracted from leucaena leaflets, then quantified by HPLC. Each sample set contained a minimum of 4 biological replicates.

Treatment with various day lengths

Two-month-old leucaena seedlings were grown under hydroponic conditions for 5 days at 25 ± 2 °C under 16/8 h, 24/0 h or 0/24 h light/dark photoperiods. Mimosine was extracted and quantified from leucaena leaflets. In another experiment, 2-month-old leucaena seedlings were grown for 2 weeks at 25 ± 2 °C with a 16/8 h light/dark photoperiod under either white or purple light. Mimosine was extracted and quantified from the leaflets of treated leucaena seedlings. Each sample set contained at least 4 biological replicates.

Maceration of leucaena leaflets

One gram samples of mature leucaena leaflets were macerated in 20 mL of 0.1 N HCl at pH 1.8 (acidic solvent) or 0.1 M Tris-HCl at pH 8.0 (alkaline solvent) using a mortar and pestle. The macerated leucaena leaflets were incubated at room temperature and mimosine was quantified from the leaflet extract at 5, 10, 15 and 960 min after initial maceration. In another experiment, 1 g samples of mature leucaena leaflets were macerated in a mortar and pestle containing 20 mL of 0.1 M Tris-HCl buffered to pH 8.0 and containing water, 5 mM EDTA or 5 mM hydroxylamine. The macerated leaflet extracts were incubated overnight at room temperature and then mimosine and 3H4P were quantified in the leaflet extracts. The mimosine concentrations are shown as a percentage of fresh weight. Experimental sets were performed in triplicate.

Results

Mimosine and 3-hydroxy-4-pyridone (3H4P) concentrations in leucaena foliage

Among the various parts of leucaena foliage, mimosine concentration was highest in young shoot tips, which contained up to 22.2% mimosine on a dry weight (DW) basis followed by older shoot tips with 14.7% mimosine DW. Among the different leaflet sizes, younger and smaller leaflets contained a higher mimosine concentration than mature and larger leaflets (Figure 1).

Mimosine and 3H4P concentrations in fallen red leucaena leaflets were 0.30% and 0.11% DW, respectively, which were lower than those of fallen green leaflets (Table 1, Figure 2). Fresh yellowish leaflets contained significantly less mimosine (1.4% DW) than fresh normal green leaflets (6.4% DW). Dried normal green leaflets had the lowest mimosine concentrations (0.1% DW) of all the leaflets tested. These results suggest that under certain conditions, a significant portion of the mimosine in leucaena leaflets is degraded to 3H4P.

Concentrations of mimosine in the top sections of the stems were 0.47% mimosine (DW basis), which was significantly higher than for the middle and lower sections (Figure 3). The total mimosine concentration in the stems, including all sections, was 0.18% DW. These results indicate that the mimosine concentration is highest in the youngest and actively growing parts of the stem.

Mimosine concentrations in leucaena seedlings grown in NaCl solution

The mimosine concentrations in leucaeana leaflets extracted at 0 and 3 days of NaCl treatment remained relatively low at 2.44 and 2.37% DW, respectively. However, at 6 days of treatment, the mimosine concentrations had increased significantly (3.25% DW) and remained relatively high throughout the rest of the treatment time (Figure 4). This suggests that mimosine synthesis and accumulation may increase under saline conditions, possibly as a stress response.

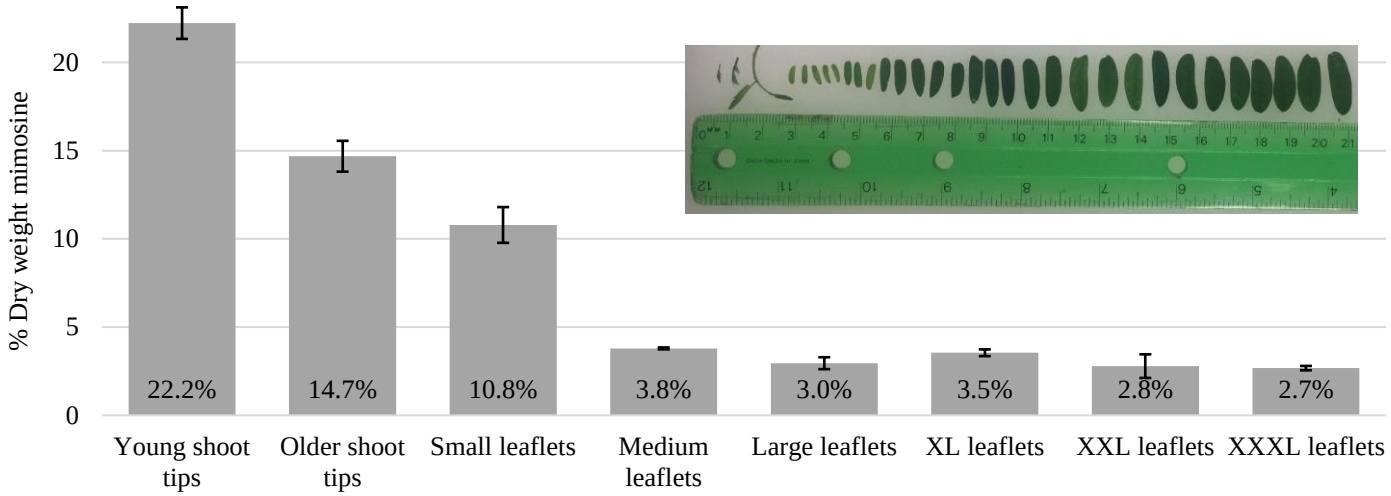

Figure 1. Mimosine concentrations in adult leucaena leaflets of various sizes and ages. Young shoot tips (shoot tip Y) contained higher concentrations of mimosine than older shoot tips (shoot tip O). Mimosine concentrations in leaflets appear to be correlated with leaflet size and age. Small and young leaflets contained significantly higher concentrations of mimosine than medium, large, extra-large (XL), extra-extra-large (XXL) and extra-extra-extra-large (XXXL) leaflets. Data are shown as a percentage of dry weight. Error bars indicate standard error from 3 replicates.

Table 1. Mimosine and 3-hydroxy-4-pyridone concentrations in various types of mature leucaena leaflets.

Leaflet type	Mimosine (% DW)	3H4P (% DW)
a) Fallen red leaflets	0.3	0.11
b) Fallen green leaflets	0.63	0.13
c) Fresh yellowish leaflets	1.4	<0.001
d) Fresh normal green leaflets	6.4	<0.001
e) Oven-dried normal green leaflets	0.10	0.38

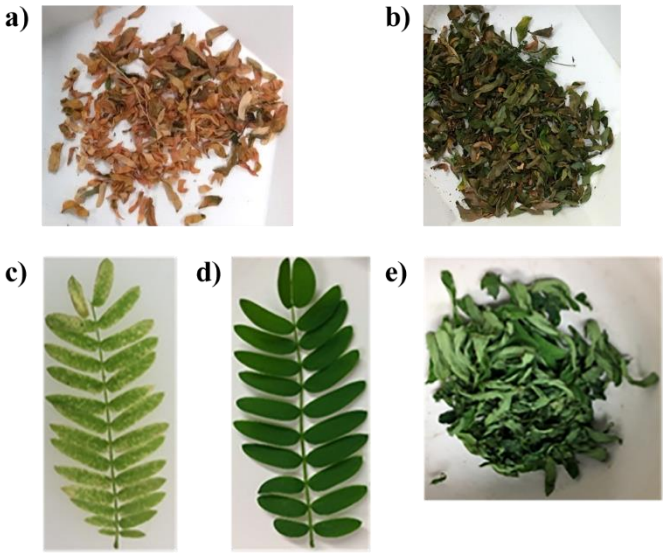

Figure 2. Mature leucaena leaflets: **a)** fallen red leaflets; **b)** fallen green leaflets; **c)** fresh yellowish leaflets; **d)** fresh normal green leaflets; and **e)** oven-dried normal green leaflets.

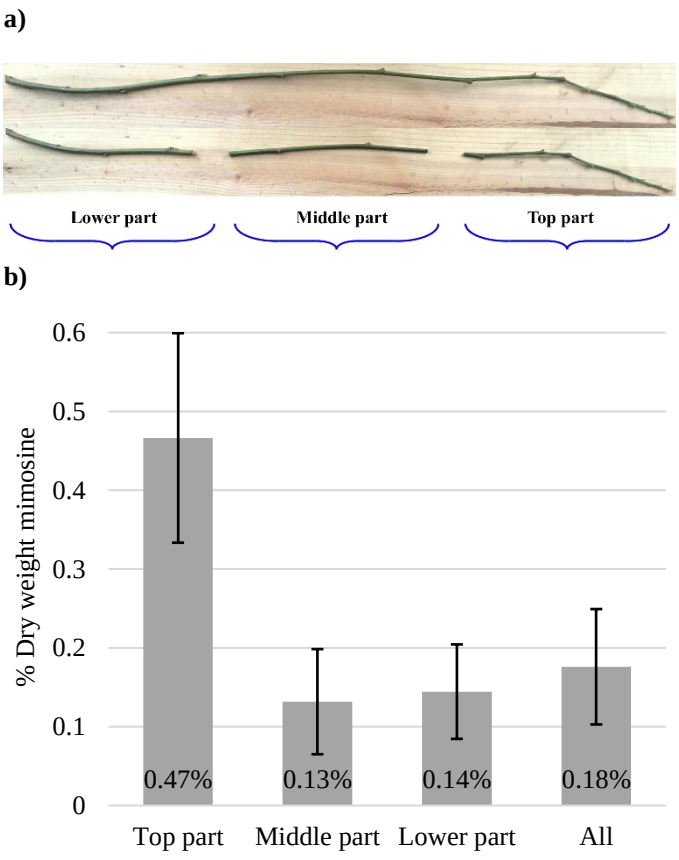

Figure 3. **a)** Mature leucaena stem divided into top, middle and lower sections; and **b)** Mimosine concentrations in mature leucaena stem sections. Data are shown as a percentage of dry weight. Error bars indicate standard error from 3 biological replicates.

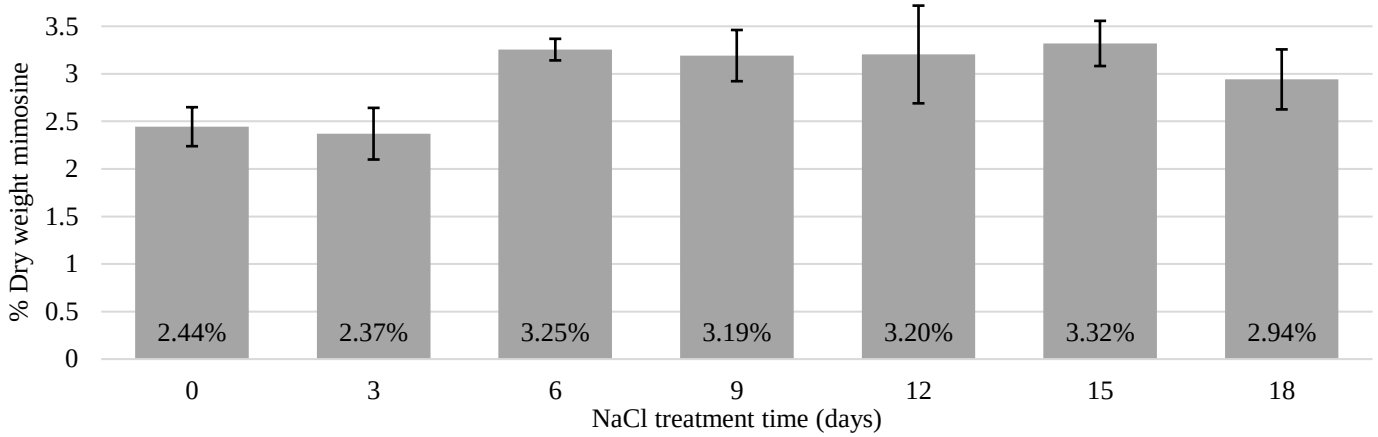

Figure 4. Mimosine concentrations in leucaena leaflets treated for 18 days with 300 mM NaCl. At 0 and 3 days of NaCl treatment, the mimosine concentration remained relatively low; however, from 6 to 18 days of treatment, the mimosine concentration had increased and remained relatively high. Data are shown as a percentage of dry weight. Error bars indicate standard error from minimum of 6 biological replicates.

Mimosine concentrations in leucaena seedlings after growing for 1 week in the presence of metallic salts

Treatment of leucaena seedlings with 10 mM $FeCl_3$ or 10 mM $ZnSO_4$ had no significant effects on the mimosine concentrations in leucaena leaflets when compared with the untreated controls (Figure 5). However, leaflets of leucaena seedlings treated with $CaCl_2$ had a lower mimosine concentration than the untreated controls. These results indicate that synthesis and accumulation of mimosine may not be a stress response following exposure to metallic ions like Fe^{3+} and Zn^{2+}. Degradation of mimosine or inhibition of the synthesis of mimosine may be a response by leucaena to exposure to excessive Ca^{2+}.

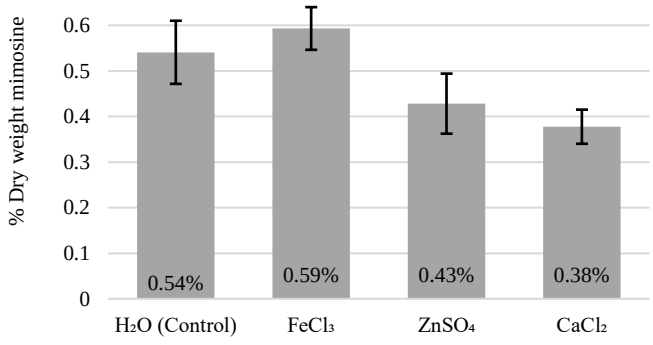

Figure 5. Mimosine concentrations in leucaena leaflets treated for 1 week with 10 mM metallic salts. Treatment with $FeCl_3$ and $ZnSO_4$ did not significantly change the mimosine concentrations in leucaena leaflets relative to the control. However, treatment of seedlings with $CaCl_2$ lowered mimosine concentration relative to the control. Data are shown as a percentage of dry weight. Error bars indicate standard error from minimum 4 biological replicates.

Mimosine concentrations in leucaena seedlings after exposure to various light treatments

Interestingly, 2-month-old seedlings exposed to 5 days of complete darkness and 5 days of total light had higher mimosine concentrations than the control plants, which were exposed to normal light/dark photoperiods (16/8 h, light/dark; Figure 6a). This suggests that excessive light or dark may stimulate mimosine synthesis and accumulation in leucaena. In the other experiment, 3-month-old leucaena seedlings grown under white light had higher mimosine concentrations (2.71% DM) than plants grown under purple light (1.97% DM) (Figure 6b). These results indicate that duration of light exposure and light color can affect the mimosine concentrations in leucaena plants.

Mimosine concentrations in leucaena leaflets after maceration

Figure 7a shows that when leucaena leaflets were macerated and incubated in solvent buffered to pH 1.8, mimosine concentrations remained high. However, when leaflets were macerated and incubated in solvent buffered to pH 8.0, mimosine concentrations decreased significantly over time. These results indicate that maceration of leaflets induces degradation of mimosine, possibly due to the release of the mimosinase enzyme from the chloroplast. Figure 7b shows that maceration solvents containing hydroxylamine contained significantly more mimosine than solvents containing water or EDTA. These results indicate that the mimosinase enzyme is inhibited by hydroxylamine but not by EDTA.

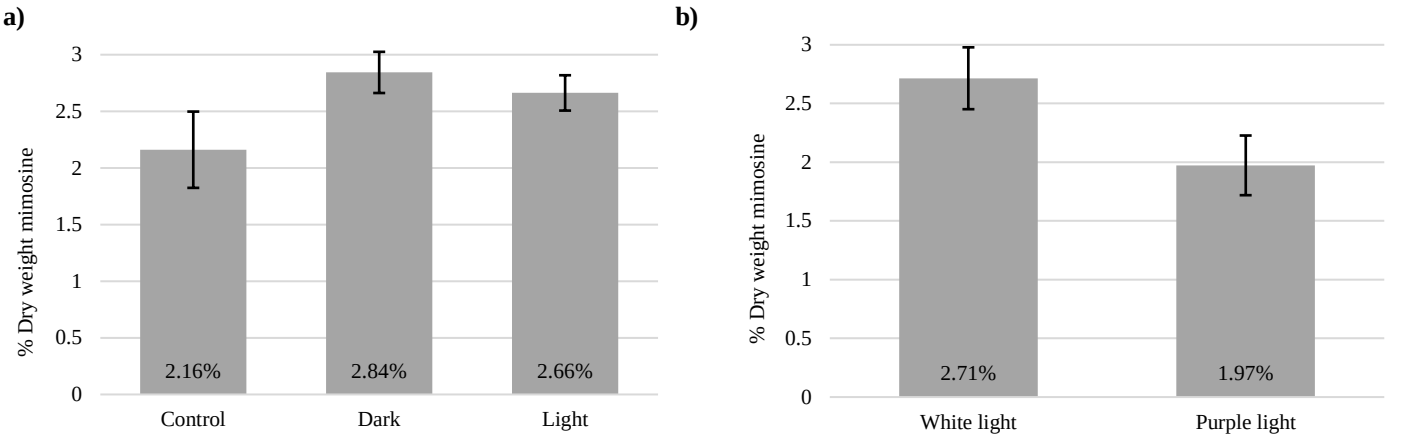

Figure 6. **a)** Mimosine concentrations in leucaena seedlings grown for 5 days with 16/8 h (control), 0/24 h (dark) or 24/0 h (light) light/dark photoperiods; and **b)** Mimosine concentrations in leucaena leaflets from seedlings grown for 2 weeks with 16/8 h light/dark photoperiods under white or purple light. Leucaena leaflets contained a higher mimosine concentration when grown under white light versus purple light. Data are shown as a percentage of dry weight. Error bars indicate standard error from minimum 4 biological replicates.

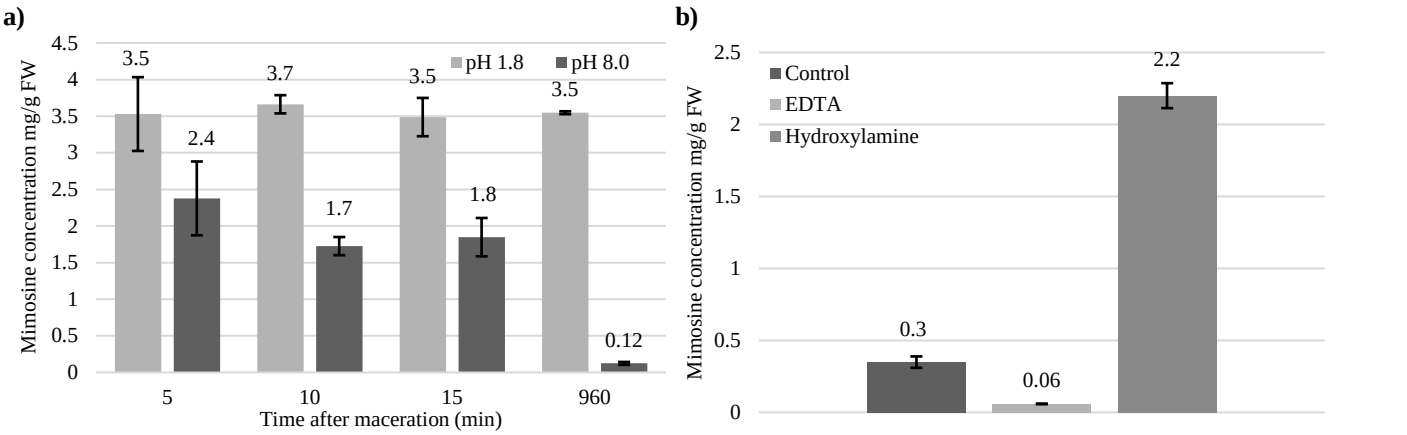

Figure 7. a) Mimosine concentrations in leucaena leaflet extracts after maceration in solvents buffered to pH 1.8 or pH 8.0 and incubated for 5, 10, 15 and 960 min. The mimosine concentrations of leaflet extracts decreased when it was macerated in solvent buffered to pH 8.0, but did not change when macerated in solvent buffered to pH 1.8; and **b)** Mimosine concentrations in leucaena leaflet extracts after maceration in solvent buffered to pH 8.0 and then incubated overnight. The mimosine concentrations remained high in solvents containing hydroxylamine, but not in solvents containing water or EDTA. Data are shown as a proportion of fresh weight (mg/g). Error bars indicate standard error from 3 replicates.

Discussion

Mimosine concentration in leucaena leaflets appears to be correlated with the size and age of the tissue. Young shoot tips, small leaflets and growing stems contained a higher mimosine concentration than larger leaflets and older portions of the stem. Mimosine and its degradation product, 3H4P, are known to have antimicrobial, nematicidal and insecticidal properties (Anitha et al. 2005; Nguyen et al. 2015; Xuan et al. 2016). A high mimosine concentration in young and actively growing portions of leucaena may be an evolutionary adaptation to protect it from browsers, and pest and pathogen attack. Herbicidal properties of mimosine have been studied and it has been shown to inhibit germination of rice, wheat and sicklepod seeds (Prasad and Subhashini 1994; Xuan et al. 2006; Williams and Hoagland 2007). Defoliation of leucaena leaflets, which contain both mimosine and 3H4P, may be a strategy to release these compounds into the soil as a means of inhibiting pathogens and preventing the growth of potential plant competitors.

The increase in leaf mimosine concentrations in leucaena seedlings grown in media treated with 300 mM NaCl relative to the untreated controls at 6–18 days of treatment may be an adaptation to salt or osmotic stress. NaCl can change the osmotic pressure of plant roots and

induce drought-like conditions. In order to prevent water loss due to drought stress, plants accumulate neutral solute compounds called osmolytes (Nahar et al. 2016), which include carbohydrates, polyhydric alcohols, methylamines and free amino acids like valine, proline, isoleucine and aspartic acid (Burg and Ferraris 2008). Mimosine may serve as an osmolyte to prevent water loss, when leucaena is under osmotic stress.

Surprisingly, the mimosine concentrations in leucaena seedlings grown for 5 days in complete darkness (0/24 h light/dark photoperiod) were higher than those in control plants (16/8 h light/dark photoperiod). Darkness as an extreme light condition can induce leaf senescence, which can lead to a decrease in proteins, photosynthetic activity and chlorophyll (Fujiki et al. 2005; Song et al. 2014). Soudry et al. (2005) found that both detached and attached *Arabidopsis* leaves had increased amino acid concentrations during senescence and suggested that the increased free amino acids may be a result of proteolysis. The increased mimosine concentrations in leucaena seedlings exposed to darkness may be the result of leaf senescence. Although mimosine is a non-protein amino acid, it is synthesized from O-acetylserine (OAS) and 3H4P in a reaction catalyzed by mimosine/cysteine synthase (Yafuso et al. 2014). During leaf senescence, proteolysis may be induced, possibly resulting in increased free serine levels, which could cause a rise in OAS levels, leading to an increase in the mimosine concentration. Increasing the mimosine concentration during prolonged darkness may be a strategy by leucaena to accumulate additional metabolites that can be utilized at a later time.

Leaflets that have fallen from plants and dried out, and oven-dried leucaena leaflets both contained significantly less mimosine than fresh leaflets. Under these conditions, mimosine is likely to be degraded by mimosinase, resulting in a lower mimosine concentration. Similarly, maceration of leucaena leaflets in an alkaline-buffered solvent caused a decrease in the mimosine concentration. This decrease is also possibly due to mimosine degradation by mimosinase after being released from the chloroplast upon maceration. Mimosinase is a PLP-dependent carbon-nitrogen lyase that degrades mimosine into 3H4P, pyruvate and ammonia (Negi et al. 2014). Mimosinase has high enzyme activity at pH 8.0 and very low activity below pH 6.0 (Negi and Borthakur 2016). This would explain why maceration of leucaena leaflets in solvent buffered to pH 8.0 resulted in significantly lower mimosine concentrations than in leaflets macerated in solvent buffered to pH 1.8. This study shows that either drying leucaena leaflets or macerating them in an alkaline solution can significantly reduce the mimosine concentration in leucaena foliage. For farmers concerned about mimosine toxicity, utilizing one of these methods may help to lower the mimosine concentration in leucaena foliage, which could also lead to an increase in the nutrient profile of leucaena used for fodder. Honda and Borthakur (2019) identified a number of genes that were highly expressed in the foliage of leucaena compared with the roots and postulated that these genes may contribute to the nutrient richness of leucaena foliage.

The mimosine concentration in leucaena is affected by environmental conditions, indicating that mimosine synthesis, degradation and accumulation fluctuate with environmental conditions. Negi et al. (2014) postulated that mimosine serves as a carbon and nitrogen reserve, which is accumulated during conditions of high nutrient availability, and is degraded during periods of low nutrient availability, such as during drought. As previously mentioned, mimosine may serve leucaena as an osmolyte to help it retain moisture under osmotic stress. Rodrigues-Corrêa et al. (2019) found that mimosine accumulates in giant leucaena in response to various stress elicitors. In the same study, they found that mimosine had the ability to quench free radicals and limit oxidative damage in foliar discs of bean plants. Osmotic and oxidative stresses are secondary stresses induced by primary biotic and abiotic stresses (Wang et al. 2003). The changes in the mimosine concentrations in leucaena may be a response mechanism to help it cope with the secondary stresses induced as a result of a primary stress. One possible explanation for the ability of leucaena to tolerate a wide range of environmental conditions might be that it produces large amounts of mimosine, which may serve multiple roles in stress tolerance.

Acknowledgment

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(Note of the editors: All hyperlinks were verified 29 April 2019.)

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ILC2018 Keynote paper*

Incorporating leucaena into goat production systems

Integrando leucaena en sistemas de producción de caprinos

FRANCES C. COWLEY AND ROMANA ROSCHINSKY

School of Environmental and Rural Science, University of New England, Armidale, NSW, Australia. www.une.edu.au

Abstract

The integration of leucaena into goat production systems in the tropics and subtropics is reviewed. Goats are well adapted to leucaena, and able to be productive on diets containing up to 100% leucaena as a result of bacterial and hepatic detoxification. Incorporation of leucaena into goat production systems can improve liveweight gains, milk production, worm control and reproduction. Successful feeding systems for goats can be based on both grazed silvopastoral systems and cut-and-carry intensive systems, although there is a lack of farming systems research examining the integration of leucaena into goat production systems, or documentation of the practicalities of these practices.

Keywords: *Caprus aegagrus hircus*, cut-and-carry, grazing, silvopastoral systems, tree legumes.

Resumen

La integración de leucaena en los sistemas de producción de caprinos en el trópico y subtrópico es revisado en este trabajo. Los caprinos están bien adaptados al consumo de leucaena y son capaces de ser productivos en dietas que contienen hasta un 100% de leucaena como resultado de la detoxificación bacteriana y hepática. La incorporación de leucaena en los sistemas de producción caprina tiene el potencial de mejorar las ganancias de peso vivo, la producción de leche, el control de parásitos internos y la reproducción. Sistemas de alimentación exitosos para caprinos pueden basarse tanto en pastoreo en sistemas silvopastoriles como en sistemas intensivos de corte y acarreo. Sin embargo, hay una escasa investigación sobre sistemas agropecuarios que examinen la integración de leucaena en los sistemas de producción caprina, y de documentación de aspectos prácticos de esta integración.

Palabras clave: *Caprus aegagrus hircus*, corte y acarreo, leguminosas arbóreas, pastoreo, sistemas silvopastoriles.

Introduction

Goat production systems in tropical and subtropical regions of Southeast Asia, Africa and South America are often characterized by a high seasonal variability of forage biomass availability and low protein concentration in herbaceous pasture species, preventing goats from meeting maintenance and production requirements (Mtenga and Shoo 1990; Clavero and Razz 2003) and from expressing their genetic potential (Leketa 2011). The high protein concentration in *Leucaena leucocephala* (leucaena) makes it a valuable feed resource for ruminants in tropical and subtropical conditions to fill these gaps. The nutritional benefits of feeding leucaena to ruminants extend to goats, and have been well studied, as has the toxicology of leucaena's most significant secondary compound, mimosine, and its primary metabolites, the dihydroxypyridones (DHP). However, the practicalities of using leucaena in goat management systems have been poorly documented.

Goats have physical and behavioral characteristics which cause them to rely much more on the browsing of shrubs than other ruminants, and grazing leucaena would appear to be a natural fit for goat production systems. However, this production system brings with it the risk of ring-barking of trees. Therefore, in several countries, leucaena is integrated into goat production systems as a cut-and-carry fodder.

Leucaena is fed to goats across a large range of tropical and subtropical regions. An analysis of research articles published on the topic reveals that the majority of research

Correspondence: F.C. Cowley, School of Environmental and Rural Science, University of New England, Armidale, NSW 2350, Australia. Email: fcowley@une.edu.au

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on feeding leucaena to goats is published in Asia, Africa and South America, regions in which leucaena is commonly used as a feed resource for goats (Table 1).

Table 1. Countries in which research on goats and leucaena has been recently published. Derived from Scopus database 2013–2018.

Region/Country	No of publications
Asia	
India	10
Thailand	4
Vietnam	4
Malaysia	2
Japan	3
Philippines	2
Africa	
Nigeria	5
Mozambique	2
Cameroon	1
Cote d'Ivoire	1
Ethiopia	1
Gabon	1
South Africa	1
Tanzania	1
Uganda	1
Americas	
Venezuela	4
Mexico	2
United States	3
Pacific	
Samoa	3
Australia	2
Middle East	
Israel	1
Palestine	1
Europe	
Germany	2
Netherlands	2
Belgium	1
Sweden	1

Adaptation to leucaena toxicity

Some of the earliest research on leucaena toxicity was reported on goats fed leucaena. The first experiments indicating a role for rumen bacterial metabolism of mimosine and DHP were conducted in goats in Hawaii and Australia (Jones 1981; Jones and Megarrity 1983). *Synergistes jonesii* was first isolated from the rumen fluid of goats (Jones 1981; Allison et al. 1992) and subsequently formed the main focus of leucaena detoxification research. Surveys of the presence of *S. jonesii* in leucaena-fed goat populations in Southeast Asia indicates that it is not ubiquitously present at high levels in goats fed leucaena, ranging from 32% of sampled goats in Thailand to 67% in Vietnam and 95% in eastern Indonesia, despite the long history of feeding leucaena to goats and cattle in these locations (McSweeney et al. 2014). More recent developments in understanding of leucaena toxicity have shown that not only are there a wide range of bacterial genera able to detoxify mimosine and DHP in the rumen (Derakhshani et al. 2016) but also hepatic conjugation pathways play an important role in the detoxification of DHP (Halliday 2018). *Synergistes jonesii* has now been shown to be indigenous to all ruminants, whether or not previously exposed to leucaena, although often at very low levels, which are insufficient to completely detoxify all DHP, especially where intake levels of leucaena are high. DHP, which is not completely detoxified from 3,4-DHP to the less toxic 2,3-DHP by rumen bacteria, can be conjugated in the liver by the process of glucoronidation. It is concluded that by utilizing these 2 pathways of detoxification, goats are highly productive on sole diets of leucaena without the need for inoculation with *S. jonesii*; however adaptation to leucaena feeding is required in order to upregulate both pathways of detoxification (Halliday 2018). Unfortunately, most research concerned with feeding value and production responses of goats fed leucaena do not report on the animals' past history of leucaena consumption, inoculation status, current efficacy of detoxification, and in many cases, experimental diet adaptation protocols. All of these factors could interact with the intake and productivity of goats fed leucaena.

Feeding value of leucaena for goats

Leucaena can be fed to goats as an alternative source or cheaper substitute for conventional protein feed supplements (e.g. oilseed cake meals), which are often expensive or unavailable in more remote or extensive production systems (Clavero and Razz 2003; Leketa 2011). The presentation of leucaena and proportion of stem in the diet will significantly affect the results of leucaena-feeding experiments. Unfortunately, many experiments either feed stripped leaves only, which is unlikely to be representative of grazing or hand-feeding production systems, or do not specify the proportion of stem in the diet.

As part of a goat ration, leucaena provides both protein and roughage. Reports indicate a sole diet of leucaena fed to goats has digestibility coefficients for dry matter (DM) of 57–66% (form not specified), organic matter of 59–67%, crude protein (CP) of 62% (Mtenga and Shoo 1990) to 65% (Girdhar et al. 1991), and total digestible nutrient concentration of 59% (Girdhar et al. 1991). Chemical composition of leucaena is superior to that of other leguminous feeds, as leaf contains more CP (27.5%) and

lower neutral detergent fiber (NDF, 24.4%) than lucerne (*Medicago sativa*), lablab (*Lablab purpureus*) and desmanthus (*Desmanthus bicornutus*) (20.3–21.5% CP and 23.6–36.9% NDF; Kanani et al. 2006). In vitro dry matter digestibility of leucaena (47%, including stem <2 mm diameter at a rate of 33% of feed on offer) was similar to that of pigeon pea (48%, including stem at a rate of 36% of feed on offer), but lower than that of the tree legume sesbania (62%, including stem at a rate of 43% of feed on offer), most likely as a result of sesbania's high acid detergent fiber (ADF) concentration (38%) (Karachi and Zengo 1997).

The high protein concentration and digestibility of leucaena increase the digestibility and CP concentration of the whole diet, which in many tropical goat production systems is likely to be quite low. Combining tropical, low-protein grass species or other forage resources with leucaena can increase DM intake. Including the leaves and soft stems of leucaena (stem proportion unspecified) in a basal diet of *Hymenachne pseudointerrupta* and concentrate (20% CP, 10.2 MJ ME/kg DM) resulted in partial substitution of the more digestible leucaena for the low-quality grass, increasing total DM intake (although without a recorded effect on digestibility of DM, organic matter or CP; Rahman et al. 2015). Mtenga and Shoo (1990) also reported that Tanzanian goats reduced their intake of *Chloris gayana* hay as amount of leucaena leaf on offer increased, but increased total intake on a liveweight basis. In that experiment, increasing leucaena on offer up to 100% of the diet had no effect on DM digestibility of the diet (Mtenga and Shoo 1990). However in some cases leucaena supplementation can increase DM intake without substitution. Supplementing a hay mixture of S*etaria palidefusca* and *Imperata cylindrica* with leucaena leaves resulted in only a low-level (non-significant) of substitution, but increased intakes of DM, CP and energy in goats (Tshibangu et al. 2015). When leaves and petioles of leucaena (29.6% CP) were fed to Barbari goats as a supplement to a rice straw diet, the resulting very high CP intake (6.8 g/kg $W^{0.75}$) caused an increase in the intake of the straw portion of the diet (Dutta et al. 1999).

In some cases responses to feeding leucaena can exceed responses to vegetable protein meals. Isocaloric and iso-nitrogenous total mixed rations (TMR), formulated with either leucaena or a mix of soybean, cottonseed and sunflower meals and fed to castrated male Saanen goats, had similar CP concentrations and digestibility of dry matter, organic matter, CP, NDF and ADF, but the leucaena TMR resulted in a DM intake 40% higher on a liveweight basis (Leketa 2011). In that experiment, the increased DM intake did not correspond with a difference in digestible organic matter or CP intake on a liveweight basis (Leketa 2011). However there are instances of leucaena supplementation decreasing DM intake in goats, which may be related to poor adaptation to leucaena toxicoses.

Production responses to leucaena-based diets

Integration of leucaena into rations has demonstrated positive production responses in dairy goats. Grazing dairy goats on leucaena has led to a significant increase in milk yields (Clavero and Razz 2003). For example, with an additional 2 h of browsing leucaena in addition to pasture feeding, crossbred Saanen-Anglo Nubian goats in Venezuela yielded 101.4 kg total milk compared with 66 kg for goats fed on pasture alone (Clavero and Razz 2003). When hammer-milled leucaena leaves and stems (proportions not reported) replaced full-fat soybean meal and partially replaced sunflower oil meal and cottonseed meal in an isonitrogenous total mixed ration, there was no reduction in milk yields or milk protein and fat concentrations, but liveweight gains during lactation increased, indicating that it could contribute to reducing ration cost for dairy goats (Leketa 2011).

When leucaena was fed as a cut-and-carry supplement to a grazing diet for goats, liveweight gains increased by up to 150% in the dry season and 50% in the wet season (Karachi and Zengo 1997) over the unsupplemented control. Goats adapted by upregulated rumen bacterial and hepatic detoxification pathways to consuming leucaena achieved growth rates of 41 g/d on 100% leucaena diets, outperforming 50% leucaena:50% natural grass diets (23 g/d), 50% *Gliricidia sepium*:50% grass (15 g/d) or a 100% gliricidia diet (22 g/d) (Halliday 2018). Adejumo and Ademosun (1991) provided a more complicated view of high leucaena rations for goats. Their research found consistent decreases in total DM intake as the proportion of leucaena leaf and stalk (removed from stems) in a *Panicum maximum*-leucaena diet increased up to 80%. After 10 weeks of feeding, goats fed the 80% leucaena diet began to show symptoms of leucaena toxicity, including hair loss and excessive salivation. In contrast, in 2 trials Halliday (2018) fed goats, adapted to leucaena, a sole leucaena diet for 7 weeks and 10 weeks, respectively, and observed no clinical signs of toxicity or any reduction in intake.

While leucaena has been successfully fed as a supplement for breeding does, results are somewhat equivocal. When leucaena leaves were fed with *Calliandra calothyrus* leaves as a supplement to does grazing natural pasture, there was a reduction in abortions and an increase in kid birth weights and weaning weights (Pamo et al. 2006). Although goats grazed on leucaena and natural pasture silvopastoral systems had lower conception rates than does grazed on natural pasture alone, the products of pregnancy (foetus and

placenta) and foetal growth rates were increased (Akingbade et al. 2001). A subsequent experiment found mixed results in terms of multiple births for the silvopastoral system, but with a greater weight gain during pregnancy, and improved kidding rates, there was a benefit to the introduction of leucaena into the grazed breeding system (Akingbade et al. 2004).

Leucaena compares favorably with commercial protein concentrates and other legumes as a protein supplement for goats. When goats grazed a leucaena fodder bank (23.5% CP, 38.6% NDF) for 2 hours per day in addition to a *Cenchrus ciliaris* pasture (8.6% CP, 56.0% NDF), increases in average daily milk yields were the same as for goats supplemented with 300 g concentrate/hd/d (20.0% CP; Clavero and Razz 2003). When fed as a supplement to a sudangrass (*Sorghum bicolor*, 7.8% CP, 63.2% NDF, 36.7% ADF) diet, the leucaena (27.5% CP, 24.4% NDF, 13.4% ADF)-grass diet produced higher average daily gains, forage gain efficiency and intakes of legume than diets supplemented with lucerne (20.3% CP, 34.2% NDF, 26.5% ADF), lablab (21.5% CP, 36.9% NDF, 24.9% ADF) or desmanthus (21.5% CP, 23.6% NDF, 12.6% ADF) as a result of its higher CP and lower fiber concentrations (Kanani et al. 2006).

The positive production responses obtained from feeding leucaena to goats are not surprising, and correlate with similar documented benefits for cattle. However, there has been little research comparing nutritional responses of goats fed on leucaena with responses in sheep or cattle, and it would be interesting to know whether the smaller, but more digestively efficient rumen of the goat is able to gain more nutritional benefit from leucaena than cattle.

Leucaena feeding systems for goats

While the benefits of feeding leucaena to goats have been well documented, there is a pressing need for information on the optimal method for including leucaena in goat production systems. Mohammadabadi and Jolazadeh (2017) suggest that extensively grazed, intensive and small-scale production systems limited by land availability can profit from fodder trees as a feed resource for goats. Much of the published literature on the use of leucaena for goat production necessarily entails animal house experimentation, using harvested leucaena, often stripped to leaves only, or processed into hay, meal or pellets, and as such is unlikely to be representative of commercial or smallholder goat production systems. While there are a range of options for inclusion of leucaena in goat production systems, there has been little documentation of the benefits and pitfalls, including economic implications, of various approaches to practical implementation.

Grazing and silvopastoral systems

Goats are noted browsers, exhibiting a preference for sourcing their feed from shrubs at head height and above, rather than from grazed grass at foot. They have physical characteristics, including a prehensile tongue, the ability to stand bipedally and a mobile upper lip, which allow them to forage easily from trees and shrubs, such as leucaena (Sumberg 1985). Goats therefore seem to be compatible with grazed leucaena systems. Grazed alleys of leucaena under-sown with grasses in silvopastoral systems are a common production system for cattle, and have potential to be extended to goats. Leucaena shrubs are planted in dense rows with pastures or crops in the inter-row spaces. A rotationally grazed fallow system has also been proposed, consisting of 3–5 years of alley cropping between stands of leucaena, during which there is no grazing, followed by 2–3 years of grazing leucaena during a cropping fallow (Sumberg 1985).

Management of leucaena silvopastoral systems for goats is dependent on the pasture composition and breed of goat; however, a range of stocking rates have been tested, with positive production results (Table 2).

Table 2. Goat productivity under a range of leucaena silvopastoral systems and stocking rates.

Pasture under leucaena	Stocking rate (head/ha)	Average daily gain (g/hd/d)	Gain (kg/ha)	Reference
Unspecified, but invaded by *Eragrostis* spp. and *Sporobolus* spp.	11.5	45–117	28–94	Morris and du Toit (1998)
	15	71–94	60–99	
	20	60–112	66–158	
Andropogon gayanus, *Panicum maximum*, *Cynodon* spp.	73	17	43	Carvalho et al. (2017)
Cenchrus ciliaris with fodder bank of leucaena	16	48 (+ milk production)	92 (+ milk production)	Clavero and Razz (2003)

The preference of goats for leucaena or grass in silvopastoral systems is likely to be dependent on the grass species planted in the silvopastoral system, availability of grass and leucaena, season, nutritional requirements of the goats and management of the leucaena. Unfortunately, most research papers do not report biomass availability of grass or leucaena, or management of the shrub stand. This had led to a wide range of reported preferences for leucaena in silvopastoral systems (Table 3).

In alley grazing systems, preference for, and time spent browsing leucaena, will be dependent on the availability and quality of both leucaena and the understorey grass. Time spent grazing leucaena is related to the available biomass of the understorey grass. Time spent browsing leucaena increased from 24 to 40% of total grazing time as pasture height was decreased by mowing (removing the effect of quality) and pasture biomass was reduced from 597 to 312 kg/ha (Orihuela and Solano 1999). The relatively high proportion of time spent browsing leucaena (55%) reported by Ketshabile (2008) is likely to be related to the relatively low quality of the grass (5.9% CP, 78.0% NDF, 52.1% ADF) compared with the leucaena (21.7% CP, 33.1% NDF, 22.6% ADF).

Goats tend to rely more on grass during the wet season (Sumberg 1985), when its quality and quantity would be greatest. Meanwhile, browse vegetation accumulates on the leucaena, which is available to be increasingly utilized by the goats as the dry season deepens, and the biomass available in the pasture becomes limited (Sumberg 1985). When availability of grass herbage was limited during the dry season in a mixed cropping and silvopastoral system, goats were far more willing to shift their grazing preferences to leucaena browse than were sheep, which instead increased their intake of low quality millet stubble (Dicko and Sikena 1992). When grazing a diverse pasture of 18 grass species and 18 herbaceous or shrubby legume species (including a stand of leucaena), sheep and goats showed a preference for legumes/leucaena over grass, whereas cattle spent more time grazing grass than legumes (which included leucaena, Singh et al. 1997). All tested animal species increased their preference for leucaena after the monsoon season ended (Singh et al. 1997). Goats displayed a preference for legumes longer into the dry season than sheep, but during spring (late dry season) leucaena was a less-preferred species for goats, which increased their grazing effort on grass, whereas sheep and cattle continued to prefer leucaena (Singh et al. 1997). This research did not report on relative quality or availability of any of the feeds.

As the higher quality feed in a silvopastoral system, when an energy concentrate supplement is fed, leucaena intake tends to be maintained while concentrate is substituted for grass. When goats managed in a leucaena silvopastoral system were supplemented with maize concentrate, they spent on average 6 hours grazing grass to 1 hour grazing leucaena (Carvalho et al. 2017). As the level of maize supplementation was increased, the goats substituted maize for the lower quality grass (14.1% CP, 66.1% NDF, 36.2% ADF) rather than the higher quality leucaena (33.0% CP, 40.1% NDF, 25.3% ADF), with time spent grazing leucaena unaffected by the level of maize supplementation (Carvalho et al. 2017). Apart from substitution targeting the lower quality diet component, intake of leucaena may have been maintained due to its role as the main source of protein in the diet. The proportion of time that goats spent grazing leucaena was not affected by regrowth time for the leucaena (45–75 days), most likely because leucaena height and quality did not vary over this period (Costa et al. 2015).

The susceptibility of goats to gastrointestinal nematodes means that they can benefit from a diet that encourages the use of browsed shrubs such as leucaena. Browsing allows goats to avoid infection with the larval population living in the grass sward (Hoste et al. 2010). There is also the potential for goats browsing leucaena to alleviate worm burdens by consuming anthelmintic secondary compounds in leucaena,

Table 3. Preferences of goats for leucaena and grass in unsupplemented alley-planted silvopastoral grazing systems.

Understorey grass species	Leucaena stand management	Time spent browsing leucaena (% total grazing time)	Time spent grazing grass (% total grazing time)	Reference
Panicum maximum	3 m inter-row spacing, grazed at flowering stage, ~1.5 m high	55 (goats) 12 (sheep)	45 (goats) 88 (sheep)	Ketshabile (2008)
Cenchrus ciliaris	1 m inter-row spacing, planted at 6,666 plants/ha, ~1.5 m high, continuously pruned	33	67	Orihuela and Solano (1999)[1]
Andropogon gayanus, *Panicum maximum* and *Cynodon* spp.	1.9 m inter-row spacing, planted at 1,999 plants/ha	15	85	Costa et al. (2015); Carvalho et al. (2017)

[1]Mean time. Time spent browsing increased with decreasing grass availability.

although evidence of the ability of goats to self-medicate with leucaena has not been established (Hoste et al. 2010; Ventura-Cordero et al. 2018). The high condensed tannin (CT) concentration in leucaena can indirectly improve resistance and resilience of goats to worm infection by increasing protein flow to the duodenum and upregulating specific immune responses to infection (Thi Mui Nguyen et al. 2005). It has also been proposed that there is a direct effect of secondary compounds, including CT, on hatch rate and larval development in goats (Thi Mui Nguyen et al. 2005). Protein extracts from leucaena seeds have a demonstrated ovicidal effect on *Haemonchus contortus* eggs collected from goats (Soares et al. 2015). Goats fed a basal diet of rice straw supplemented with harvested leucaena foliage in an animal house experiment had worm egg counts 15–35%, and coccidian oocyst counts 25–85%, of those in goats supplemented with grasses (Nguyen Kim Lin et al. 2003).

There are several practical concerns in grazing leucaena systems with goats. Bark stripping or ring-barking is frequently raised as a risk in grazing goats on leucaena (Sumberg 1985); however, documentation of the extent and implications of this problem is scarce. Morris and du Toit (1998) noted some stripping of bark by goats during the late summer in South Africa, as did Goetsch et al. (2014), although this did not kill any of the trees. Bark stripping has been reported even when leaves were plentiful (Muir et al. 1991). When bark is stripped around the entire circumference of a stem or trunk, die-off occurs above the point of the damage, but the plant survives and new growth continues below the point of damage. In the study of Muir et al. (1991), on average 72% of the circumference of damaged branches was stripped. Plants with only part of the stem circumference stripped re-grew bark over the stripped area, in some cases completely. As damage increased, the number of branches below 30 cm height increased, and the number of branches above this height decreased and leaf biomass distribution followed the same pattern. The suitability of leucaena for coppicing indicates that leucaena may be resilient to ring-barking damage. However the comparative productivity of the plants under cutting or grazing by goats has not been tested.

Up-rooting of browsed shrubs can be a problem with other grazed tree legumes established with stake techniques, such as gliricidia (Sumberg 1985), but establishing leucaena from seed has prevented this problem in research in Nigeria. Seed-establishment may also prevent branch damage to browsed trees, as branches naturally grow lower along leader stems, rather than from the top of stakes (Sumberg 1985). Coppicing also promotes low branching, and can improve access to foliage, as well as preventing branch damage. Tree height can become a restriction for grazing goats to access leucaena browse. Wild bush buck (*Tragelaphus scriptus*) and Nguni and Boer goats (*Capra hircus*) have preferred grazing heights of less than 0.5 m (Haschick and Kerley 1996; du Plessis et al. 2004) and maximum grazing heights of 122 (Boer goats) and 166 (bush buck) cm, respectively (Haschick and Kerley 1996). This constraint can be addressed by coppicing, bending down stems of narrow trunks (Sumberg 1985), cutting branches above 1.2 m when necessary (Muir et al. 1991) and choice of leucaena variety.

The economic benefits and costs of fallow leucaena-goat alley-grazing systems compare favorably with alley-cropping systems, such as maize-cassava, in West Africa (Sumberg 1985). Grazing fallow regrowth reduces capital investment and management issues associated with planted pastures (Sumberg 1985). A comparison of the use of alley-grown leucaena as goat feed with its use as a green mulch for a maize crop in Western Tanzania predicted a 50% benefit of feeding the leucaena to goats (Karachi and Zengo 1997). In general, there are limited system or whole-farm gross margin analyses of these systems, and there are many key issues which need research.

Cut-and-carry feeding systems

Leucaena is fed to goats mainly as fresh material in cut-and-carry feeding systems, which are flexible, and labor- and resource-efficient (Sumberg 1985; Palmer et al. 2010). In many cases, the leucaena inputs to these systems are derived from alley-cropping systems, similar to the silvopastoral systems described above. One hectare of alley-cropped land can yield 4 tonnes of leucaena foliage, of which 25% can be removed without reducing crop yields, sustaining 3 does and their offspring on a sole leucaena diet (Upton 1985). In other cases leucaena is obtained from planted fodder banks, or harvested from wild-grown shrubs.

In cut-and-carry feeding systems leucaena can act as a protein supplement or form the whole of the diet. Farmers feed it chopped or directly offer branches to goats which are intensively housed, tethered or free-grazed. Leucaena can also be fed (sun)dried (Mtenga and Shoo 1990) and processed into leaf meal (Mohammadabadi and Jolazadeh 2017) or as leaf protein concentrate (Farinu et al. 1992), although these systems are less often practiced by farmers. Leaves, stems and bark can all be fed to goats. Bark has been shown to be palatable to goats although the total amount of bark needs to be limited to avoid a reduction in nutritive value of the total ration (Palmer et al. 2010). When fed cut-and-wilted material, goats have displayed a strong preference for leucaena over other tree forages *Albizzia lebbek*, *Gliricidia sepium* and *Tamarindus indica* (Mtenga et al. 1994). When fed to housed goats, leucaena can be presented as whole or chopped branches. Feeding in troughs is

common, but hanging branches in bunches or laying them on racks above the goats' heads caters to their natural browsing instinct, while reducing the potential for nematode larval infection from feces which can fall into ground-level troughs.

Conclusions

Goats have a natural inclination to browse woody shrubs, are frequently raised in tropical and low-nutrition production systems and are well suited to integration with leucaena. They can adapt to leucaena toxicosis through bacterial and hepatic detoxification pathways, which permits productivity gains by the addition of leucaena, up to 100% of the diet. Despite a large body of research demonstrating the benefits of leucaena in various goat diets and production systems, there is a lack of information identifying the differences between goats and other ruminant species in the use and utilization of leucaena. Concerns that goats are unsuited to silvopastoral systems owing to the risks from ring-barking persist, although the ability of leucaena to survive and resprout from low on the stem, as well as documentation of successful grazed leucaena systems, suggests that these fears may be overstated. However, there is little documentation regarding the practicalities or economics of successful extensive or intensive goat production systems that include leucaena. A priority for future work is farming systems research examining the integration of goats and leucaena, including in crop-livestock systems.

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(Note of the editors: All hyperlinks were verified 24 April 2019.)

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Energy supplements for leucaena
Suplementación energética para leucaena

KAREN HARPER[1], SIMON P. QUIGLEY[1], RISA ANTARI[2], DAHLANUDDIN[3], TANDA SAHAT PANJAITAN[4], MARSETYO[5] AND DENNIS P. POPPI[1]

[1]*School of Agriculture and Food Sciences, The University of Queensland, Gatton, QLD, Australia.* agriculture.uq.edu.au
[2]*Beef Cattle Research Institute, Grati, Indonesia.*
[3]*University of Mataram, Lombok, Indonesia.* unram.ac.id
[4]*The East Nusa Tenggara Assessment Institute for Agriculture Technology, Kupang, Indonesia.* ntt.litbang.pertanian.go.id
[5]*Tadulako University, Palu, Central Sulawesi, Indonesia.* untad.ac.id

Abstract

Leucaena can be fed as the sole diet to fattening cattle without nutritional problems and it will promote high liveweight gains. The high crude protein concentration in leucaena suggests that energy supplements, which are readily fermented in the rumen, could be used to capture the excess rumen degradable protein and provide more microbial protein and metabolizable energy to the animal, further increasing liveweight gain or milk production. This approach has been tested in grazing cattle and also in cut-and-carry systems in Australia and Indonesia. In both systems, production (liveweight gain or milk production) increased with the addition of supplements containing large amounts of fermentable metabolizable energy. The substitution of the basal diet (leucaena or leucaena mixed with grass or crop residues) by the supplement also means that more animals can be carried in the system for a set amount or area of leucaena. The same principles would apply to any tree legume-based system. Energy supplements can come in many forms, viz. fermentable starch (cereal grains and cassava), sugars (molasses), pectins (soybean hulls and pulps) and fibre (rice bran, cassava bagasse), but they have not been compared for their efficacy nor for their economic benefit, if any, in these systems.

Keywords: Cut-and-carry systems, forage utilization, legume-energy combinations, liveweight gains, substitution effects.

Resumen

La leucaena se puede usar como dieta única para ganado de engorde sin que se presenten problemas nutricionales, resultando en altos aumentos de peso vivo. La alta concentración de proteína cruda en la leucaena sugiere que suplementos energéticos fácilmente fermentados en el rumen podrían ser usados para capturar el exceso de proteína degradable en el rumen y proporcionar más proteína microbiana y energía metabolizable al animal, aumentando aún más la ganancia de peso vivo o la producción de leche. Esta estrategia ha sido probada en sistemas de pastoreo y de corte y acarreo en Australia e Indonesia. En ambos sistemas, la producción (ganancia de peso vivo o producción de leche) aumentó con la adición de suplementos que contenían grandes cantidades de energía metabolizable fermentable. La sustitución de la dieta base (leucaena o leucaena mezclada con pasto o con residuos de cultivos) por el suplemento también significa que se pueden mantener más animales en el sistema por una cantidad o área determinada de leucaena. Los mismos principios se aplicarían a cualquier sistema basado en árboles leguminosos. Los suplementos energéticos pueden ser de muchas formas, tales como almidón fermentable (granos de cereales y yuca), azúcares (melaza), pectinas (cáscaras y pulpa de soya) y fibra (salvado de arroz, bagazo de yuca), pero aún no se han comparado por su eficacia ni por su eventual beneficio económico en estos sistemas.

Palabras clave: Combinación leguminosas-energía, efecto de substitución, ganancia de peso vivo, sistemas de corte y acarreo, utilización de forraje.

Correspondence: K. Harper, School of Agriculture and Food Science, The University of Queensland, Gatton, QLD 4343, Australia.
Email: karen.harper@uq.edu.au

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Introduction

Leucaena has a high crude protein (CP) concentration and high dry matter digestibility (DMD) and is used as a protein supplement or legume forage within grazing systems. As well as providing a source of CP, especially during the dry season or when straw residues from cropping systems are fed, it is also a source of extra energy. It can also be used as a sole forage in grazing or cut-and-carry systems, especially in Asia and Latin America, and produces good liveweight gains (LWG). Panjaitan et al. (2014) and Dahlanuddin et al. (2014) have shown that Bali cattle fed solely on leucaena in a cut-and-carry system gained 0.47–0.61 kg/d, which is close to the genetic potential for growth of this cattle species (Figure 1). Under this feeding regime, the CP consumed is in excess of the CP requirements of all classes of ruminants.

The excess CP may be viewed as wasteful or energetically costly as the ruminant catabolizes and excretes the excess N in the form of urea, largely in the urine. However, having a diet with excess CP is not in itself a physiological problem for the animal, as ruminants have evolved to cope with diets containing a wide range of various nutrients including CP and/or N. Nutritional principles define the excess or deficit of N in the rumen for the microbes, or amino acids at the tissue level for cell metabolism. These feeding standards demonstrate that leucaena provides excess rumen degradable protein (RDP) and hence excess N for rumen microbes given the fermentable metabolizable energy (ME) of leucaena and also provides an excess of absorbed amino acids. While animals can cope with this situation quite readily, nutritionists often assess things on a 'requirement' basis and define excess and deficit scenarios as 'problems', which need to be fixed by balancing the diet. Rather than being a 'problem', this scenario presents an opportunity to make more efficient use of the high-protein forage in the leucaena.

Figure 1. Bali bulls in a traditional fattening system with 100% leucaena in West Sumbawa District, Indonesia.

The opportunity

An excess of RDP in the rumen provides the opportunity to increase microbial crude protein (MCP) production by increasing the supply of fermentable ME to microflora within the rumen. The excess of absorbed amino acids within the small intestine (metabolizable protein, MP) presents an opportunity to increase animal performance by providing additional ME. How might this be exploited?

Supplementing a sole leucaena diet with a highly fermentable ME source with low CP concentration is one possible option. This approach would increase MCP production and also increase ME supply through absorbed volatile fatty acids from the rumen and possibly absorbed glucose from the small intestine depending on the energy substrates that are used. Possible energy sources are starches, sugars and pectins, i.e. the common carbohydrates which are rapidly fermented in the rumen, as well as other fermentable fiber sources. Starch is provided by the common cereal grains such as wheat, barley, sorghum and corn, which have moderate CP concentrations (10–14%), plus other less commonly used feedstuffs such as cassava. Devendra (1977) quotes composition of cassava tubers of about 35% starch, about 90% nitrogen free extract, 11.9–14.6 MJ ME/kg DM and 2–4% CP, while Heuzé et al. (2016) suggest a much higher starch concentration of 69–89% DM and an ME value of 11.5–12.9 MJ/kg DM (mean of 12.2) for ruminants. Pectin is found in by-products such as soybean hulls and pulps such as citrus pulp, pineapple pulp and tomato pulp, all by-products from other industries. The main sugar sources are molasses (high in ME and low in CP) and root crops such as fodder beet. From a nutritional perspective, a supplement high in ME and low in CP, e.g. cassava or molasses, is optimal, but other common cereals such as wheat, barley, sorghum or corn or the various pulps can also be used. Similarly, a case can be made for other by-products which have reasonable fermentable ME values such as rice bran, cereal bran and pollard. Availability and price will determine the energy source chosen.

The principle in such an approach is to target the excess RDP and provide a fermentable substrate containing starch, pectin, sugars or digestible fiber. This will enable capture of the excess RDP within the rumen and an increase in MCP production, in addition to an increase in ME supply. The extra MP may not be required but the response curve of LWG to extra MP is curvilinear (Black and Griffiths 1975) and, although the extra MP is used with low efficiency for growth, there will still be a LWG response. Poppi (1990) showed in New Zealand that LWG of lambs still increased in response to extra MP despite CP values in

excess of 25%. This comes about by the animal using the surplus amino acids as a source of energy as well as a source of amino acids. If MP is limiting, there will be a huge response but the calculations for leucaena alone show that the primary limiting nutrient is ME.

The practical response

If the above approach is followed, viz. providing extra fermentable ME because RDP is in excess of requirements of the rumen microflora with a sole leucaena diet, a response in LWG is expected. This is not the only benefit expected since, as the level of an energy supplement is increased, there is a substitution effect on intake of the basal diet (McLennan et al. 2017). This means that, when the energy supplement is fed, the amount of leucaena consumed declines. The practical significance is that a limited amount of leucaena can be used to feed more animals when a mixed diet of leucaena plus an energy supplement is fed than if a sole leucaena diet is fed. This has important implications for cut-and-carry systems and for grazing systems based on leucaena, where dry matter yield of leucaena is the limiting component in the system. In practical terms, a cut-and-carry farmer or one with a grazing system can support more animals on a limited area of leucaena by feeding an energy supplement. Such an approach has been used by Petty et al. (1998) and Petty and Poppi (2012) in grazing systems in Australia, and by Panjaitan and Dahlanuddin (unpublished data) in cut-and-carry systems in Indonesia and Timor Leste. In places such as Australia where land is less limiting, it may be simpler and more economic to plant a larger area of leucaena.

The evidence: Grazing systems

Grazing systems do not use a leucaena-only pasture base. The early work of Quirk et al. (1990) in south Queensland showed that annual LWG could be increased from 90 kg/steer on native pasture to 205 kg/steer on native pasture with leucaena planted in rows 3 m apart. Current recommendations in Australia are to plant leucaena at 8–10 m inter-row spacings to increase the total biomass production within the system by increasing grass growth (S. Buck pers. comm.).

The principle of energy supplementation could also be applied in these grazing systems, both to utilize the high RDP from leucaena and to increase overall ME intake. Petty et al. (1998) and Petty and Poppi (2012) grazed cattle on a pangola (*Digitaria eriantha*)-leucaena pasture and supplemented them with increasing levels of maize grain or molasses up to 10 g DM/kg LW/d. In both experiments significant responses in LWG (up to 0.35 kg/hd/d) to molasses were obtained but responses to maize grain occurred only in the first study. Both studies showed a similar substitution effect whereby leucaena intake declined at high levels of maize or molasses sup plementation. This substitution effect is very important as it allows more stock to be supported on a limited area or quantity of leucaena. The economics of this practice needs careful evaluation as supplementation is rarely profitable in these grazing situations in Australia. The response curves developed by Petty et al. (1998) and Petty and Poppi (2012) provide a methodology to assess various situations economically.

A similar experiment in Brazil with goats grazing a leucaena-grass system and supplemented with increasing amounts of maize grain produced almost identical results to the studies with cattle in northern Australia (Carvalho et al. 2017). They compared levels of maize grain supplement up to 13 g DM/kg LW/d, and LWG of the goats increased from 18 g/d without supplement to 67 g/d at the highest supplement level in a linear fashion, allowing stocking rate to be increased in response to the substitution effect.

The evidence: Cut-and-carry systems

There is a large number of reports whereby feeding leucaena or other tree legumes in a cut-and-carry system increased intake and LWG or milk production of ruminant animals (Poppi and Norton 1995). Legumes are used to supplement forages with low (e.g. straws) to moderate (e.g. elephant grass) CP concentration, all with relatively low DMD. In all cases there is a curvilinear response in intake and LWG with a rapid increase up to an inclusion level of approximately 10 g DM/kg LW/d and a slower increase to a plateau at higher levels.

As with grazing systems, leucaena is usually a supplement and not the sole forage. In these cases the results are similar to those from the grazing systems outlined above, i.e. increases in total intake and LWG. Flores et al. (1979) supplemented dairy cows grazing nitrogen-fertilized Rhodes grass with leucaena up to 3.5 g DM/kg LW/d and increased milk production from 9.6 to 10.3 kg/d. Where an energy supplement has been used with the leucaena the results mirror those of the grazing systems, viz. a further increase in LWG combined with a substitution effect. For example, Muinga et al. (1995) reported milk production of dairy cows fed Napier grass (5.1 kg milk/d) or Napier grass supplemented with 2 kg DM leucaena/d (5.5 kg/d) or 2 kg DM leucaena plus 1 kg DM maize bran/d (6.5 kg/d) in a cut-and-carry system. Quigley et al. (2009) conducted a series of experiments to evaluate LWG of

weaner Bali cattle fed grasses, and supplemented with tree legumes and protein meals, alone and in combination with energy supplements. LWG was increased from 0.1–0.2 kg/d (grass only) to >0.5 kg/d, with the highest gains in weaners fed leucaena ad libitum with 10 g maize or 10 g rice bran/kg LW/d (0.56 and 0.61 kg/d, respectively). This was comparable with gains by weaners fed a high CP (18%) concentrate ration (0.65 kg/d). To basal diets of either corn stover or elephant grass hay fed ad libitum to Bali bulls, Marsetyo et al. (2012) fed a supplement of gliricidia (*Gliricidia sepium*) at 10 g DM/kg LW/d. Gliricidia supplementation at this level increased LWG from 0.17–0.23 to 0.28–0.31 kg/d. There are many such examples in the literature and from this conference.

Supplementing with leucaena will markedly increase animal performance where the basal diet is low in CP (<7% CP) as it will stimulate the microflora and increase DM intake, but can also increase performance where the basal diet is higher in CP (>7% CP), although responses would be smaller. The latter effect is moderated by the comparative DMD of both feed sources. The major effect of leucaena in these systems is to increase overall ME intake but the accompanying increase in MP intake is also important. Providing an energy supplement with the grass-leucaena mix will usually further increase LWG. As with grasses, quality of leucaena can vary markedly depending on the proportion of leaf consumed. Some cut-and-carry systems feed the whole plant in an intact form and animals select mostly leaf and leave a large amount of stem residue, so the CP % and DMD of the leucaena consumed is high. Other systems put the leaf and stem through a chopper to minimize waste and the overall CP % and DMD of the chopped mixture is reduced by the large amount of stem so animals have difficulty selecting a high quality diet. Hand-plucked leucaena leaf can have CP of 30% and DMD of 61.7% (Petty et al. 1998), while Karachi (1998) showed leaf averaged 25% CP and 58% DMD and stem averaged 13% CP and 36% DMD. The large difference in these parameters between leaf and stem highlights the difference in quality of feed selected by animals fed whole branches and those fed chopped material.

There are fewer reports where leucaena (or other tree legumes) was the sole diet of fattening animals and where an energy supplement has been fed with leucaena. Budisantoso (cited by Quigley et al. 2009) demonstrated an increase in LWG of Bali bulls from 0.42 kg/d (leucaena alone) to 0.61 kg/d (leucaena plus maize) or 0.56 kg/d (leucaena plus rice bran), both supplements constituting about 34% of the final ration. Partial substitution occurred as intake of leucaena with the supplemented rations was 15–23% lower than when leucaena was fed alone. Dahlanuddin et al. (2014) compared leucaena, sesbania (*Sesbania sesban*) and gliricidia when fed as the sole diet and found leucaena and sesbania resulted in much higher LWG than gliricidia. With all tree legumes, animals responded to an energy supplement usually in the form of rice bran or maize grain. These findings support the theoretical arguments outlined in the early section of this paper. Dahlanuddin et al. (2014) showed LWGs of 0.34 kg/d in Bali bulls fed sesbania alone and 0.43 kg/d with sesbania plus rice bran, while Panjiatan et al. (2018) demonstrated LWGs of 0.33 kg/d in Bali bulls fed native grass alone and 0.53 kg/d when the native grass was supplemented with sesbania plus maize grain. Bali bulls fed leucaena plus maize grain achieved 0.66 kg/d (Dahlanuddin et al. 2018).

Differences in response to additional energy could depend on the form of energy supplement (e.g. starch vs. sugars vs. pectin vs. highly digestible fiber) but such comparisons are limited, e.g. Budisantoso (cited by Quigley et al. 2009). More recently a series of unpublished experiments (Kusmartono and F. Cowley pers. comm.; Dahlanuddin and Panjaitan unpublished data) have shown that cassava and cassava bagasse ('onggok') may be used as effective energy sources but high levels of inclusion (>50% cassava or bagasse in the ration) can depress intake and LWG. This phenomenon does not appear to be related to starch alone, as similar studies, where grain was fed with grass-based diets, showed no depression in LWG but a substitution effect of the grain on hay intake (McLennan et al. 2017).

Leucaena and most tree legumes have a CP concentration of 20–25% with leaf plus small amounts of stem, and up to 30% CP in leaf alone, a DM digestibility of approximately 60% and a degradability of 66% (Bamualim et al. 1980; 1984a; 1984b). The RDP:DOM ratio is 188–236 g RDP/kg DOM compared with a rumen microbial requirement of 130 g RDP/kg DOM (PISC 2007). A supplement or total mixed ration of 50% leucaena and 50% energy supplement would supply approximately 177 g RDP/kg DOM for a cereal grain energy source and 138 g RDP/kg DOM for a cassava tuber energy source, both of which are close to the requirements of rumen microbes for N and should maximize MCP production. We were unable to find a comparison of these energy sources in such a situation. As both energy sources are readily available at very competitive prices (depending on country and region), there is an urgent need to evaluate them under these feeding systems. While the role here is primarily to provide fermentable ME for the high RDP from leucaena, when used at very high levels (or at total mixed ration formulation), this proportional mix provides both RDP and high ME to the animal. With the substitution effect it would enable a limited amount of leucaena to be used to feed more

animals. These formulations have application in fattening diets in Asia and could have a role in enhancing the use of leucaena in northern Australia. The use of cassava with leucaena would be a system similar to that already studied by Petty et al. (1998) and Petty and Poppi (2012) with maize or molasses. Cassava and its by-products could also be used with leucaena or any other protein source (e.g. algae, forage legumes or protein meals) to devise supplements or total mixed rations for use in backgrounding live export cattle or finishing cattle out of season. In all these circumstances biological and economic responses need to be evaluated, as economic analysis may show that feeding leucaena alone is the most economic. A whole-of-enterprise analysis is required rather than an individual animal response as greater throughput (more animals) may be of more interest to smallholders wishing to increase cattle numbers and overall profit.

The case for cassava-leucaena systems

The mix of cassava or its by-products with leucaena has many advantages from a systems perspective as outlined above and meets the nutrient requirements of both rumen microflora and the ruminant animal (e.g. fattening bulls). The current inter-row system used in Australia, Asia and Latin America combines leucaena and grass, which is often low in ME. Maize or cassava could be substituted for grass in the inter-row of a leucaena system, especially those systems in Asia (Figure 2), and the grain, stover, cassava tubers and cassava leaves could be utilized. Cassava tubers are very high in ME (see above) and low in CP. This would substantially increase the total DM yield from the system and mixing the whole cassava tubers with leucaena in a total mixed ration would provide a high quality product. Feeding a 50:50 mixture of cassava tuber and leucaena ad libitum or at 16 g/kg LW/d to Bali bulls resulted in LWGs of 0.57 and 0.42 kg/d (Dahlanuddin unpublished data; Panjaitan unpublished data). When 40–50% cassava was fed with a range of protein sources (gliricidia, copra meal or palm kernel cake), LWGs of 0.39 kg/d in Bali bulls (Marsetyo unpublished data), 0.75 kg/d in Madura bulls (Kusmartono and F. Cowley pers. comm.) and 1.39 kg/d in Limousin/Ongole crossbred bulls (Retnaningrum and Kusmartono pers. comm.) were achieved. Commercial feedlot rations fed to Ongole bulls using cassava and protein meals achieved 0.8 kg/d (Antari et al. 2012) and, in a village supplement experiment, 0.82 kg/d (Ratnawati et al. 2015). These values are very high and approaching or equivalent to the highest recorded LWGs for most of these cattle breeds. One might expect that using leucaena as the protein source would produce similar results.

Figure 2. Leucaena cv. Tarramba inter-row maize in West Sumbawa District, Indonesia.

Conclusions

Leucaena leaves have high CP and ME concentrations. There are no detrimental nutritional consequences of such a high CP concentration in the diet, and the only issues associated with feeding a 100% leucaena diet are mimosine and DHP toxicity. The high CP concentration creates opportunities for using leucaena in fattening systems. The traditional approach is to use it as a supplement to low-CP dry season pastures (grazing scenario) or crop residues (various stovers) with positive effects on LWG. In Australia this has evolved into year-round grazing (leucaena-grass pasture) providing a higher quality overall diet than grass alone and supporting higher stocking rates. With total mixed rations in cut-and-carry systems leucaena can be combined with an ingredient with high ME, such as cereal grains, pulps, bran or cassava, resulting in a high quality mixture which promotes improved LWGs. The advantage of feeding a leucaena-energy source mixture is that a given amount of leucaena can be used to fatten more animals and increase cash flow of the smallholder farmer.

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(Note of the editors: All hyperlinks were verified 29 April 2019.)

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ILC2018 Keynote paper*

Evaluating crude protein concentration of leucaena forage and the dietary legume content selected by cattle grazing leucaena and C4 grasses in northern Australia

Evaluando la concentración de proteína cruda de forraje de leucaena y la proporción de la leguminosa en la dieta seleccionada por ganado pastoreando mezclas de leucaena con gramíneas C4 en el norte de Australia

KYLIE HOPKINS[1], MAREE BOWEN[1], ROB DIXON[2] AND DAVID REID[1]

[1]*Department of Agriculture and Fisheries, Queensland Government, Parkhurst, QLD, Australia.* daf.qld.gov.au
[2]*Queensland Alliance for Agriculture and Food Innovation (QAAFI), The University of Queensland, Parkhurst, QLD, Australia.* qaafi.uq.edu.au

Abstract

In Australia's central and southern Queensland regions, *Leucaena leucocephala*-grass pastures produce substantially more beef and higher profits than grass-only pastures and annual forage crops. Near infrared reflectance spectroscopy (NIRS) provides a rapid and cost-effective approach to assessing quality of available forage as well as the quality of the diet selected by cattle, but existing calibrations have not been comprehensively validated for leucaena-grass pastures. This study examined the reliability of existing northern Australian calibrations for NIRS to predict the crude protein (CP) concentration of the edible fraction of the leucaena plant, and the proportion of leucaena in the diet of grazing cattle. Samples of edible leucaena and cattle feces were analyzed by NIRS and the predictions plotted in a linear regression and fitted to a 1:1 line with Dumas analysis of CP for leucaena forage, and mass spectrometry of $\delta^{13}C$ for cattle feces. Results demonstrated that prediction of the CP concentration of leucaena forage and the proportion of leucaena in the diet of grazing cattle using current broad northern Australian NIRS forage calibrations were associated with substantial error. However, it is likely that these errors can be reduced with the inclusion in the calibration data set of more samples representing leucaena forage and feces of cattle grazing leucaena from varying locations, seasonal conditions and management strategies.

Keywords: Diet quality, forage quality, near infrared reflectance spectroscopy, tree legumes, tropical pastures.

Resumen

En las regiones central y sur de Queensland, Australia, pasturas con *Leucaena leucocephala* y gramíneas producen sustancialmente más carne y mayores ingresos en comparación con pasturas de solo gramíneas o con cultivos forrajeros anuales. La espectroscopía de reflectancia en el infrarrojo cercano (NIRS) es un método rápido y económico para evaluar la calidad de forraje disponible, así como la calidad de la dieta seleccionada por el ganado. Sin embargo, las calibraciones disponibles no se han validado de manera exhaustiva para las pasturas de asociaciones de leucaena con gramíneas. En este estudio se examinó la confiabilidad de las calibraciones actualmente existentes en el norte de Australia para NIRS, para predecir la concentración de proteína cruda (PC) en la fracción comestible de plantas de leucaena y la proporción de leucaena en la dieta seleccionada por ganado pastando mezclas con gramíneas. Se analizaron por NIRS muestras de

Correspondence: Kylie Hopkins, 25 Yeppoon Road, Parkhurst, QLD 4702, Australia.
Email: kylie.hopkins@daf.qld.gov.au

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leucaena y de heces de ganado y se proyectaron las predicciones en una regresión lineal ajustadas a una línea 1:1 usando el método de análisis de Dumas para CP en el forraje de leucaena y la espectrometría de masas de $\delta^{13}C$ para las heces de ganado. Los resultados demostraron que usando las calibraciones de NIRS que actualmente existen para forraje en el norte de Australia, las predicciones de la concentración de PC en el forraje de leucaena y de la proporción de leucaena en la dieta del ganado en pastoreo, estaban asociadas con errores sustanciales. Sin embargo, es probable que estos errores se puedan reducir si en el conjunto de datos de calibración se incluyen muestras adicionales representativas de forraje de leucaena y de heces de animales pastoreando leucaena provenientes de diferentes lugares, condiciones estacionales y estrategias de manejo.

Palabras clave: Calidad de dieta, leguminosas arbóreas, NIRS, pastos tropicales, valor nutritivo.

Introduction

The ~123,500 ha of established *Leucaena leucocephala*-grass pastures is important to the beef industry in central and southern Queensland (Beutel et al. 2018), as it provides opportunity to substantially increase beef production and profitability compared with perennial grass pastures and other sown forages (Bowen et al. 2018). However their optimal management requires knowledge of available quantity and quality of both the leucaena and grass pasture components, especially crude protein (CP) concentration, dry matter digestibility (DMD) and the proportion of leucaena in the diet selected by grazing cattle (Bowen et al. 2015).

Near infrared reflectance spectroscopy (NIRS) provides a rapid and cost-effective approach to not only assess the quality of the forage (plant) material presented to cattle, but also the quality of the diet selected by grazing cattle by testing their feces. NIRS predictions depend on the availability of reliable and robust calibration equations appropriate to the forages and grazing systems of interest. Broad NIRS calibrations have been developed for most common pastures in northern Australia (Coates 2004; Dixon and Coates 2009), but have not been comprehensively validated for leucaena-grass pasture systems. This study examined the reliability of these northern Australian NIRS calibrations to predict the CP concentration of the edible fraction of leucaena forage and the proportion of leucaena in the diets of grazing cattle.

Materials and Methods

Samples of the leucaena forage selected by grazing cattle (leaf and stem <5 mm in diameter, considered the 'edible' fraction of leucaena forage), and feces of cattle grazing leucaena-grass pastures were collected as described by Bowen et al. (2015) from 4 commercial producer sites in the Fitzroy River Basin. These samples represented a range of environments, seasonal conditions and management strategies.

Edible leucaena forage samples (n = 31) were analyzed for CP by both wet chemistry (Dumas) and by NIRS (Dixon and Coates 2009), with CP predicted from established 'in-house' calibrations suitable for northern Australian forages (Coates and Dixon unpublished data). Fecal samples (n = 48) from cattle grazing these leucaena-grass pastures were analyzed for $\delta^{13}C$ by mass spectrometry and the proportions of C3 species in the diets calculated, with corrections for diet-tissue discrimination and differences in digestibility and $\delta^{13}C$ values between the C3 and C4 species (Bowen et al. 2018). NIRS of feces (F.NIRS) was used to predict the non-grass proportion of the diet using calibrations for northern Australian tropical pastures (Dixon and Coates 2008). Linear regressions between NIRS predictions of CP in forage and that measured by Dumas, and F.NIRS predictions of non-grass in the diet and that measured by mass spectrometry, were fitted and compared with the 1:1 line.

Results

There was a strong linear relationship between the NIRS-predicted CP concentrations of edible leucaena forage and those measured by wet chemistry (R^2 = 0.90), but the regression differed ($P<0.05$) from the 1:1 relationship (Figure 1a); samples containing >ca. 22% CP were under-predicted. The relationship between the proportion of leucaena in the diet, predicted by F.NIRS as % non-grass, and that calculated from the $\delta^{13}C$ measured by mass spectrometry, did not differ from a 1:1 line (Figure 1b), but there was considerable variation about the regression line (R^2 = 0.78).

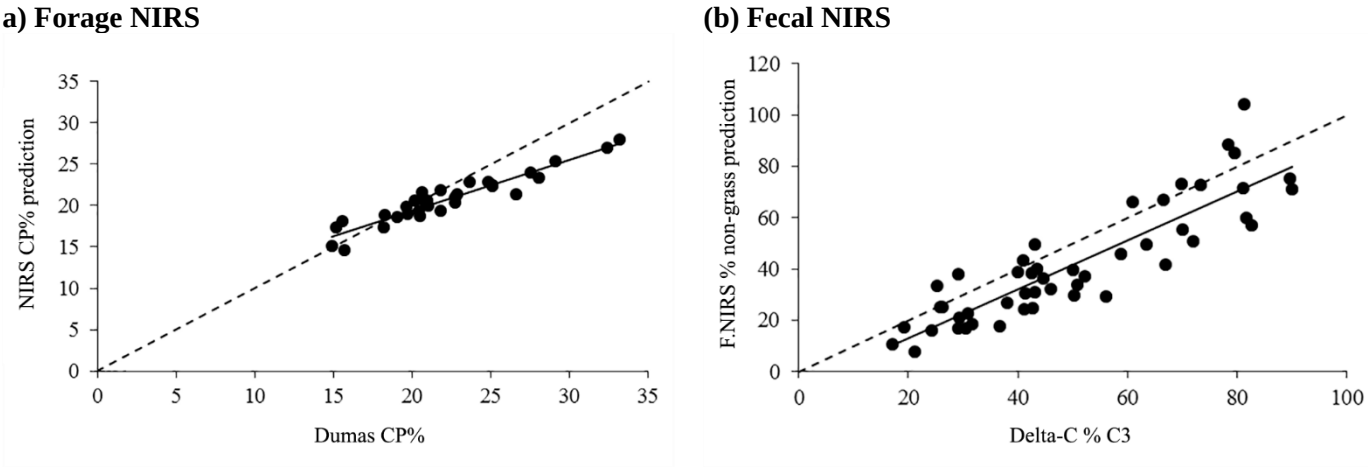

Figure 1. a) The relationship between edible leucaena CP% (Y) predicted by NIRS and that measured by Dumas (X); Y = 0.61X + 7.2 (n = 31; R^2 = 0.90); intercept >0 (P<0.05) and slope <1 (P<0.05). **b)** The relationship between proportion of leucaena in the diet (Y) predicted by F.NIRS and that calculated from $\delta^{13}C$ in feces (X): Y = 0.95X - 6.1 (n = 48; R^2 = 0.78); the relationship did not differ (P>0.05) from the 1:1 line. The 1:1 relationships are indicated by a dashed line (----).

Discussion

The broad NIRS calibration equation for forage samples used to predict the CP concentration of the 'edible' fraction of leucaena forage was developed from a large calibration data set dominated by tropical grasses and containing only a few samples of leucaena forage. Thus the observed deviation of CP% of leucaena forage as predicted by NIRS from the 1:1 relationship (Figure 1a) was not unexpected. While this error was minor for the range ca. 17–22% CP, the equation substantially underestimated the CP concentration in samples above this range. For an NIRS calibration to be reliable, it must include samples applicable to the forage type, location and season of those being analyzed. The existing calibration for northern Australian tropical pasture systems proved unsatisfactory in predicting the CP% of leucaena forage. However, inclusion of additional samples of leucaena forage into the calibration sample set, particularly those with CP outside of the range ca. 17–22%, is likely to reduce the errors associated with predicting CP% in leucaena forages containing low or high concentrations of CP. This is supported by the study of Wheeler et al. (1996) which showed that satisfactory calibrations with a validation R^2 = 0.89 can be developed for prediction of the CP concentration of leucaena forage.

The F.NIRS calibration equation used to predict the proportion of leucaena in the diet of grazing animals was based on a large sample set of feces from cattle grazing northern Australian pasture systems which included few samples (n = 9) from leucaena-grass pastures. Within that calibration set there was a close relationship between the reference and predicted values [R^2 = 0.90, standard error of cross-validation (SECV = 6.6% units)] and this calibration satisfactorily predicted the leucaena % in the diet in a previous study (R^2 = 0.92; n = 15; relative standard deviation = 8.1 % units; Dixon and Coates 2008). However in the present study, the relationship between the measured $\delta^{13}C$ reference values and those predicted by F.NIRS using the above mentioned calibration were poor with R^2 = 0.78 (Figure 1b). As discussed above for NIRS predictions of CP% in forage, it is likely that the errors in prediction of non-grass (% C3 or leucaena) content of diets of cattle grazing such pastures can be reduced by including in the calibration data set more samples representing these diets from varying locations, seasonal conditions and management strategies. It must also be noted that F.NIRS calibration sets do not currently account for the difference in digestibility between C3 and C4 forage species; it is possible that the errors in prediction may be further reduced by accounting for this factor.

Improvement of F.NIRS calibrations to predict the diet of cattle grazing leucaena-grass pastures can be expected in the future. However, until such improvements can be made to the NIRS predictions of dietary non-grass, $\delta^{13}C$ should be used for scientific experiments.

In conclusion, measurement of the CP concentration of leucaena forage using current broad northern Australian NIRS forage calibrations was associated with substantial error, when CP concentrations were above ca. 22%. In addition, measurement of the leucaena content of the diet of cattle grazing leucaena-grass pastures using F.NIRS and the current broad northern Australian F.NIRS calibration equations was associated with substantially larger errors

than those for most grass and grass-stylo pastures. Given the economic importance of leucaena-grass pastures in northern Australia and the advantages of the NIRS technology for measurement of forage and diet attributes in grazing cattle, it is important that the northern Australian NIRS calibrations are refined to more accurately and reliably measure the quality of forages and that of diets selected by cattle in leucaena-grass pasture systems.

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ILC2018 Keynote paper*

Dual use of leucaena for bioenergy and animal feed in Thailand

Uso de leucaena para bioenergía y alimentación de ganado en Tailandia

SAYAN TUDSRI[1], SONGYOS CHOTCHUTIMA[1], KARNDA NAKAMANEE[2] AND KUNN KANGWANSAICHOL[3]

[1]*Department of Agronomy, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand.* www.ku.ac.th
[2]*Department of Livestock, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.* www.moac.go.th
[3]*PTT Research and Technology Institute, PPT Public Company Limited, Ayutthaya, Thailand.* www.pttplc.com

Abstract

Leucaena is a dual-purpose plant suitable for producing both biofuel and feed for livestock (dairy and beef cattle, buffalo and goats). It has a high woody stem yield under repeated cutting and has a suitable chemical composition for excellent heat generation on combustion. Yields of leaf, which is a by-product of this process, are also high and the leaf has high nutritive value as an animal feed. Tarramba appears the highest yielding cultivar available, and many hybrid lines show excellent potential. Plant spacing of 1 × 0.50 m is recommended with cutting not more frequently than once a year. Harvesting of the crop should be carried out as a compromise between the needs for biofuel and livestock feed. On infertile soils application of at least 750 kg triple superphosphate, 188 kg KCl and 188 kg gypsum/ha/yr is recommended. Some limitations on growing and the management of leucaena are discussed.

Keywords: Dual-purpose crops, energy production, fodder shrubs.

Resumen

Leucaena leucocephala es una planta de doble propósito que puede producir tanto biocombustible como forraje para el ganado (bovinos de carne y leche, búfalos y cabras). La biomasa leñosa, obtenida por cortes repetidos, es alta y, por su composición química, posee un elevado poder calorífico que hace a esta planta muy apta para la generación de calor vía combustión. La biomasa de hojas, un importante subproducto en la generación de biocombustible, es igualmente alta y posee un alto valor nutritivo para el ganado. Actualmente, Tarramba es el cultivar con mayor rendimiento disponible aunque muchas líneas híbridas muestran un excelente potencial. Se recomiendan siembras espaciadas de 1 × 0.50 m con cortes no más frecuentes que una vez al año. La cosecha debe llevarse a cabo teniendo en cuenta las necesidades de biocombustible y la alimentación del ganado. En suelos infértiles se recomiendan aplicaciones de al menos 750 kg de superfosfato triple, 188 kg de KCl y 188 kg de yeso/ha/año. Este trabajo también discute algunas limitaciones sobre el crecimiento y el manejo de la leucaena.

Palabras clave: Arboles forrajeros, biocombustible, cultivos de doble propósito.

Introduction

It is well known that energy resources in Thailand have declined significantly and the demand for energy is currently satisfied primarily by importing fuels such as natural gas, charcoal and oil. Alternative energy sources are needed to replace natural oil and gas, preferably renewable sources. One solution is to utilize and transform the available local biomass from trees and agricultural residues into less-expensive electrical energy. To avoid increasing the harvesting of fuel wood from local natural forests, an alternative is to establish tree farms to provide a continuous supply of woody biomass. Leucaena (*Leucaena leucocephala*) has poten-

Correspondence: Sayan Tudsri, Kasetsart University, P.O. Box 1097, Bangkok 10903, Thailand.
Email: agrsat@ku.ac.th

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tial for this purpose and as well as supplying wood for bioenergy production has the following advantages over other fast-growing trees: It can fix atmospheric nitrogen while other trees like Eucalypts cannot; and the leaves and young green stems are highly palatable and highly nutritious for animal feed. Leucaena leaves are more readily acceptable than e.g. Acacia leaves, while Eucalypt leaves are often refused by stock. Like other tree species it has a deep root system, making it drought-resistant and productive during the dry season, and it can reduce greenhouse gases through absorbing CO_2 from the atmosphere (Dalzell et al. 2006).

Growing leucaena for energy and forage

Varieties

An increasing array of cultivars and lines of leucaena are now available and many hybrids are being produced. Farmers are faced with the dilemma of what cultivars/ lines to plant.

Recommendation. Research in Thailand suggests that the recommended cultivars/lines of leucaena for combined use as biofuel and forage are Tarramba and the hybrid lines KU3, KU15, KU19, KU38, KU39, KU45, KU48 and KU56 (all KU lines were selected from F2 plants of the University of Hawaii´s KX2 hybrid). However, Tarramba is the only one with seed available at present, while seed production of the hybrids is still under investigation.

Research evidence. Tudsri et al. (2010) collected 65 leucaena lines from various parts of Thailand and some F2 hybrids from Hawaii for testing at the Kasetsart University Research Station (Pakchong) over a period of 3 years. Based on total biomass yields the 8 top-ranking lines were all hybrids (KU3, KU15, KU19, KU38, KU39, KU45, KU48 and KU56) out-yielding the native (naturalized) ecotypes and current commercial cultivars, Peru and Cunningham (Figure 1). Other trials on the same site compared cvv. Peru and Cunningham with cv. Tarramba (from Australia) and 2 hybrid lines (from Hawaii) planted at a spacing of 1 × 0.5 m and cut annually for 7 years. As shown in the other study, the introduced lines, Tarramba and the hybrids KU66 and KU19, produced more woody biomass (23.0–24.8 t DM/ha/yr), leaf dry matter (3.4–4.0 t/ha/yr) and total biomass (30.4–30.7 t DM/ha/yr) than Cunningham and Peru (Table 1; Figure 2).

In a further study, 5 varieties/lines of leucaena (Cunningham, Peru, Tarramba, KU17 and KU19) were compared at a spacing of 1 × 0.5 m and harvesting 3 years after planting (Sripongpakapun 2011). Again Tarramba had the greatest stem diameter (4.9 cm) and woody stem yield (29.6 t DM/ha/yr), while Cunningham had the lowest (3.7 cm and 11.6 t DM/ha/yr, respectively). Means for the 2 hybrid lines were intermediate but superior to Cunningham and Peru. However, leaf yields were similar in all lines (range of 0.6–1.0 t DM/ha/yr).

Figure 1. Native leucaena (left) and hybrid line (KU19) (right) 6 months after planting.

Figure 2. Cvv. Tarramba (left), Peru (middle) and Cunningham (right); plants one year old.

Spacing

Recommendation. Plant spacing for maximum yield in a dual-purpose leucaena plantation during the first 4 years after planting should not exceed 1 × 0.5 m. This provides both high stem and leaf production to satisfy high energy production and the by-product of nutritious animal fodder.

Table 1. Cumulative (7 years) leaf, woody stem and total dry matter yields, woody stem heating value and ash concentration plus leaf crude protein (CP) concentration in 5 leucaena cultivars/lines at Pakchong, Nakhon Ratchasima Province (Tudsri et al 2010, 2015; Rengsirikul et al. 2011).

Cultivar/line	Leaf (t/ha)	Woody stem (t/ha)	Total DM[1] (t/ha)	Heating value[2] (kcal/g DM)	Ash[2] (%)	Leaf CP[2] (%)
Tarramba	24.1	173.6	213.1	4.7	2.18	25.7
Cunningham	19.2	131.1	169.4	4.6	1.93	25.6
Peru	14.0	90.3	116.7	4.6	2.06	24.7
KU19	27.2	161.1	212.8	4.6	1.72	24.3
KU66	27.8	167.0	215.1	4.6	1.75	24.2

[1]Includes branches. [2]One sampling date.

Research evidence. Chotchutima et al. (2013) studied the effects of plant spacing (1 × 0.25, 1 × 0.5, 1 × 1, 1 × 1.5, 2 × 0.5 and 2 × 1 m) on growth, biomass production and wood quality of leucaena cut annually at 0.5 m above ground level during 2006–2010 at Pakchong, Nakhon Ratchasima province. Spacing had a significant effect on plant diameter at breast height and biomass yield with highest stem diameter at the widest spacing (2 × 1 m), while DM yields of leaf, stem and total biomass were highest at the narrowest spacing (1 × 0.25 m) (Table 2). Since the minimum diameter for logs required for the biomass gasification system is 2.5 cm (Arjhan et al. 2007), stems produced at the narrowest spacing may be unsuitable for this purpose. Additional disadvantages of this narrow spacing were the higher seed and planting costs than for wider spacings. The wider spacing not only requires less seed but also allows better mechanized access for weed control. The optimal plant spacing for leucaena will depend on the relative importance given to production of biofuel and leaf for livestock feeding.

Harvesting interval

Recommendation. Leucaena should not be harvested more frequently than annually to ensure satisfactory stem yields and stem diameter. At this frequency stem yields should be about 17.3 t DM/ha/yr and leaf yields about 2.3 t DM/ha/yr. The optimal time to harvest leucaena for biofuel and fodder production would appear to be early in the dry season (November) as stems and leaf dry quickly in the dry conditions, and the leaf can be used to supplement livestock during the period of poor pasture quality. Harvesting at this time allows slow dry season regrowth (November–February) followed by rapid growth with the onset of occasional rains in March–April and the wet season in May. On the other hand, optimal time for harvesting may become irrelevant since wood processing mills will require a regular supply of material.

Research evidence. Tudsri et al. (2010) reported that harvesting leucaena frequently (every 9 months) produced low yields of stems, which were thin (2.68 cm diameter), but high leaf yields, while delaying harvesting until 36 months increased main stem diameter and woody yields but markedly reduced leaf yields (Table 3). Despite the high yields of woody material with harvesting at 36 months, this strategy may not be suitable for Thai farmers who need an income annually. Therefore, the recommended initial harvesting age and inter-harvest interval for leucaena should be a compromise but at least 12 months after planting.

Fertilizer application

Like all crops, leucaena may benefit from fertilizer application depending on the particular soil type where the crop is established.

Table 2. Effects of plant spacing on stem diameter, cumulative (4 years) leaf, woody stem and total dry matter yields plus leaf crude protein (CP) concentration of cv. Tarramba at Pakchong, Nakhon Ratchasima Province (Tudsri et al. 2010; Chotchutima et al. 2013).

Spacing (m)	Stem diameter (cm)	Leaf (t/ha)	Woody stem (t/ha)	Total[1] (t/ha)	Leaf CP[2] (%)
1 × 0.25	2.6e[3]	10.4a	99.5a	119.4a	22.5a
1 × 0.50	3.1de	8.0b	93.9ab	110.0ab	23.8a
1 × 1.00	3.9bc	7.2b	79.5ab	94.5ab	22.9a
1 × 1.50	4.5ab	6.9b	73.0b	87.2b	24.1a
2 × 0.50	3.7cd	7.1b	83.0ab	98.3ab	23.2a
2 × 1.00	4.6a	7.9b	82.2ab	99.0ab	22.4a

[1]Includes branches. [2]One sampling date. [3]In a column, means followed by a common letter are not significantly different at P<0.05.

Table 3. Effects of cutting interval on stem diameter and cumulative leaf and stem dry matter yields, woody stem heating value and ash concentration plus leaf crude protein (CP) concentration of cv. Tarramba at Pakchong, Nakhon Ratchasima Province (Tudsri et al. 2010; Chotchutima 2015).

Cutting interval (months)	Stem diameter (cm)	Leaf (t/ha)	Woody stem (t/ha)	Heating value[1] (kcal/g DM)	Ash[1] (%)	Leaf CP[1] (%)
9 (8 cuts)	2.68c[2]	16.8	66.7b	4.4a	2.08a	25.2a
12 (6 cuts)	3.44b	13.8	97.0b	4.4a	1.98a	24.9a
18 (4 cuts)	4.57a	16.8	155.3a	4.5a	1.89a	24.5a
24 (3 cuts)	na[3]	12.3	166.1a	na	na	na
36 (1 cut)[4]	na	3.0	88.0	4.6a	2.01a	16.8b

[1]One sampling (at the end of first cycle). [2]In a column, means followed by a common letter are not significantly different at P<0.05. [3]na = not available. [4]Only one harvest and not included in statistical analyses except for CP, ash and heating value.

Recommendation. On Pakchong soil type, where soil P and K levels are 15 and 75 ppm, respectively, no applications of K, P and S are required. However, on infertile soils in northeast Thailand, which are grossly deficient in K (<10 ppm), P (<2 ppm) and S, applications of 188 kg KCl, 188 kg gypsum plus 750 kg triple superphosphate (TSP)/ha at planting and as annual dressings are suggested. As chemical fertilizers are expensive, it may be more cost-effective to apply animal manure at 20 t/ha (Tudsri et al. 2015).

Research evidence. In our research at the Buriram Livestock Research and Testing Station, Pakham district, Buriram Province, K was applied during the wet season each year to leucaena plants cut once a year for 4 years. All plots received the same amount of P and S. Plots receiving at least 94 kg KCl/ha produced significantly higher (>100% increase) leaf and woody stem yields than the treatment receiving no K annually (Table 4). Leucaena plants receiving no K had yellow leaves, stunted growth and some plants died (Figure 3). Therefore, application of at least 188 kg KCl/ha to leucaena at planting is recommended on infertile soils in lower northeast Thailand (Tudsri et al. 2015).

Table 4. Effects of potassium fertilizer on cumulative (4 years) leaf, woody stem and total dry matter yields plus leaf crude protein (CP) concentration of cv. Tarramba at Pakham, Buriram Province (Tudsri et al. 2015).

Fertilizer rates (kg KCl/ha/yr)	Leaf (t/ha)	Woody stem (t/ha)	Total[1] (t/ha)	Leaf CP[2] (%)
0	4.2b[3]	21.4c	28.5b	20.1
94	9.1a	42.9bc	58.2b	17.9
188	14.3a	78.2ab	101.4a	18.6
375	12.5a	86.4ab	105.7a	18.5
750	13.1a	104.7a	123.6a	19.5

[1]Includes branches. [2]One sampling date. [3]In a column, means followed by a common letter are not significantly different at P<0.05.

Figure 3. No potassium fertilizer (left) and 750 kg KCl/ha applied (right).

In another trial on the same site, significant responses of leucaena to P and S fertilizers were reported (Chotchutima et al. 2016). Over the 2 years of the study, stem diameter, leaf and woody stem DM yields increased progressively (>200 and 400% yield increases, respectively) to the highest level of TSP (750 kg/ha) applied (Table 5). Leucaena also responded to added S through increased stem diameter (59% increase) plus leaf (96%), woody stem (232%) and total dry biomass (200%) yields. Without S, leucaena yields were very low and plants appeared yellow and unhealthy (Figure 4). Therefore, applications of 750 kg TSP and 188 kg gypsum/ha/yr are recommended on these infertile soils. In contrast, on the Pakchong soil type in Pakchong district, Nakhon Ratchasima Province, there were no responses to P, K and S. Soils in these areas have P and K levels of 15 and 75 ppm, respectively.

Table 5. Effects of application of sulfur (S) and phosphorus (P) on stem diameter and cumulative (2 years) leaf and woody stem dry matter yields of cv. Tarramba at Pakham, Buriram Province (Chotchutima et al. 2016).

Fertilizer	Stem diameter (cm)	Leaf (t/ha)	Woody stem (t/ha)	Total[1] (t/ha)
S (kg gypsum/ha/yr)				
0	1.7b[2]	2.3a	7.1b	10.2b
187.5	2.7a	4.5a	23.6a	30.9a
P (kg TSP/ha/yr)				
0	1.8c	2.4c	8.7c	11.9c
94	2.1bc	3.0b	12.9bc	17.3bc
188	2.1bc	3.3b	14.4b	19.4b
375	2.3ab	3.3b	15.3b	20.5b
750	2.6a	4.9a	25.5a	33.3a

[1]Includes branches. [2]In a column, means followed by a common letter within main effects are not significantly different at P<0.05.

Figure 4. Cv. Tarramba fertilized with S (187.5 kg gypsum/ha) and P (750 kg TSP/ha) (left) and no S and P applied (right).

Heating values, ash and chemical composition of woody stems

Research evidence. Heating value of stems was similar for all cultivars/lines (4.6–4.7 kcal/g DM) (Table 1), cutting intervals of 9–36 months (4.4–4.6 kcal/g DM) (Table 3) and plant spacings (>4.5 kcal/g DM). Lewandowski and Kicherer (1997) suggested that ideally biomass used for bioenergy production should contain at least 3.35 kcal/g DM. Energy concentrations in all woody material in our studies exceeded this critical value.

Bakker and Elbersen (2005) suggested that ash concentration is critical in determining the value of plant material as a biofuel. All cultivars/lines in our studies had ash concentrations in woody stems of less than 3% regardless of age at harvest and plant spacing (Tables 1 and 3).

Concentrations of H, O, K, S and ADF had been reported by Rengsirikul et al. (2011) to be similar in all cultivars/lines tested by them, but the newly introduced cultivar/lines, Tarramba, KU19 and KU66 contained higher C concentrations than the current cultivars, Peru and Cunningham. On the contrary, concentrations of N, Ca, Mg and acid detergent lignin were higher in Tarramba, Peru and Cunningham than in KU19 and KU66, while both hybrids (KU19 and KU66) exhibited higher cellulose concentrations than the other cultivars. Low lignin concentration in the plant is an advantage for the cellulosic biomass because of the need to remove lignin (Moore et al. 2008). KU19 and KU66 are more suitable for bioethanol production than the other cultivars due to their lower lignin and higher cellulose concentrations in woody stems. N concentrations of the hybrids KU19 and KU66 are also lower than the maximum critical level for biofuel production (Obernberger et al. 2006).

Chemical composition of leaf as a by-product of biofuel production

Research evidence. The introduced varieties (Tarramba, KU19 and KU66) can provide a greater amount of wood for bioenergy production than the existing cultivars; the leaf yields and protein concentrations are also high, providing a ready source of protein for feeding livestock. Rengsirikul et al. (2011) demonstrated that CP concentrations in leaf tissue (24.2–25.7%) of these newly introduced varieties were equivalent to those of currently used cultivars, Peru and Cunningham (24.7–25.6%) (Table 1). While CP concentrations were not affected by plant spacing (Table 2), cutting interval had a direct negative effect on CP concentration. Delaying cutting from every 9 months to 36 months reduced the CP concentration from 25.2% to 16.8% (Table 3). However the latter concentration is still adequate for the minimum requirements of ruminant animals (8–12%) (Norton et al. 1994). Leucaena leaves are ideal protein supplements for animals and are fed widely. Mueuangporn et al. (2018) reported that providing a supplement of 1–1.5 kg of dry leucaena leaves to milking buffaloes fed on dry pangola grass plus a supplement of 2 kg of 16% CP concentrate increased milk yield by 5.4 kg/day (94% increase) over that of the control treatment (without leucaena supplementation). Furthermore, Maksiri et al. (2017) reported that concentrations of essential fatty acids in the form of Omega 3 in meat of goats fed forage sorghum plus leucaena were higher than in meat of those receiving a ration of forage sorghum plus meal concentrate.

Conclusion

Our research studies have revealed that growing leucaena (cv. Tarramba and hybrids) as a source of renewable energy for power generation while providing leaf material as a by-product for supplementing livestock (dairy cows, beef cattle, buffalo and goats) could be highly beneficial for Thai farmers. Tarramba appears the highest yielding of existing cultivars and a number of hybrids show great potential. Plant spacing of 1 × 0.50 m is recommended, with harvesting no more frequently than once a year. Harvesting strategies should be based on a compromise between the importance of biofuel production, fodder for livestock and the need for a regular income. Fertilizer strategy will depend on the soil types on which the crop is grown.

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ILC2018 Keynote paper*

Leucaena for paper industry in Gujarat, India: Case study

Leucaena para la industria de papel en Gujarat, India: Un estudio de caso

N.K. KHANNA[1], O.P. SHUKLA[1], M.G. GOGATE[2] AND S.L. NARKHEDE[1]

[1]*JK Paper Ltd, New Delhi, India.* www.jkpaper.com
[2]*Rtd IFS, ex-PCCF, Nagpur, Maharashtra, India.* mahaforest.gov.in

Abstract

India is one of the major producers/consumers of paper and pulp products (3–4% of global share). Approximately one-fourth of industry raw material has come from wood-based plantations from the 1990s onwards. The greatest development challenge faced by the industry since that time is sourcing robust raw material from agroforestry on private lands. Following genetic improvement of leucaena (*Leucaena leucocephala*) and realization of its potential as a multiple-use species, it was introduced into India in 1980 under an international cooperation effort with support from the Swedish International Development Cooperation Agency (SIDA). It has since spread across the country as a panacea for rural needs of fuel wood, small timber and cattle forage. The paper industry has found that it has potential as raw material for paper making. One of the largest Indian paper companies is JK Paper Ltd, which has an annual production capacity of 550,000 t/yr with 3 integrated pulp and paper plants located at Songadh (Gujarat), Rayagada (Orissa) and Kagaznagar (Telangana) producing writing and printing paper and virgin packaging boards.

This case study describes the leucaena farm forestry plantation program initiated by JK Paper Ltd, Unit CPM (Central Pulp Mills). The unit, under its agroforestry and farm forestry plantation approach, planted leucaena plantations in 2009-2010 in parts of Gujarat, Maharashtra and Madhya Pradesh States. To motivate farmers in the mill's catchment area, and to build confidence in on-farm plantations, exposure visits were arranged to Andhra Pradesh, where huge tracts of agricultural land were under leucaena plantations. As a result, to date, this unit has engaged >7,800 farmers who have established leucaena plantations covering an area of >18,400 ha. A robust plantation R&D network addressed issues such as seed treatment, seed germination, rhizobial inoculation, geometry of plantations, agro-forestry models, selection and development of high production clones, establishment of clonal seed orchards, genetic improvement through mutation techniques and hybridization programs for wood quality improvement.

Keywords: Agroforestry, breeding, hybridization, mutation, plantations, pulpwood.

Resumen

India es uno de los principales productores/consumidores de productos de papel y de pulpa de papel (3–4% del total mundial). Desde la década de 1990 en adelante aproximadamente una cuarta parte de la materia prima para la industria de papel proviene de plantaciones de árboles maderables. El mayor desafío de desarrollo que enfrenta la industria de papel desde esa época es obtener fuentes sólidas de materia prima proveniente de agroforesterías establecidas en tierras privadas. Como consecuencia de las primeras actividades de mejoramiento genético y del reconocimiento de su alto potencial como especie de uso múltiple, se introdujo en 1980 la leucaena (*Leucaena leucocephala*) en India, en el marco de una cooperación con la Agencia Sueca de Cooperación Internacional para el Desarrollo (Swedish International Development Cooperation Agency, SIDA). Desde entonces se ha extendido por todo el país respondiendo como una panacea a las necesidades rurales respecto a leña, madera de dimensiones menores y forraje para ganado. La industria del papel encontró que la leucaena tiene potencial como materia prima para la fabricación de papel. Una de las compañías de papel más grandes de la India es JK Paper Ltd con una capacidad de producción de 550,000 t/año en 3 plantas

Correspondence: O.P. Shukla, Chief General Manager (Raw Materials) - JK Paper Ltd, Unit: CPM, Fort Songadh, Tapi District, Gujarat - 393660, India. Email: opshukla@cpmjk.jkmail.com

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integradas de pulpa y papel, ubicadas en Songadh (Gujarat), Rayagada (Orissa) y Kagaznagar (Telangana), produciendo papel para escribir e imprimir, y cartón de fibra virgen.

Este estudio de caso describe el programa de plantaciones agroforestales con leucaena iniciado por JK Paper Ltd, Unidad CPM (Central Pulp Mills). En el marco de un enfoque en plantaciones agroforestales y forestales, se establecieron plantaciones de leucaena en 2009-2010 en partes de los estados de Gujarat, Maharashtra y Madhya Pradesh. Para motivar a los agricultores en el área de influencia de Central Pulp Mills, y para crear confianza en el modelo de producción agroforestal, se organizaron visitas a Andhra Pradesh, donde existen grandes extensiones de tierras agrícolas con plantaciones de leucaena. Como resultado, hasta la fecha se ha involucrado a más de 7,800 agricultores quienes establecieron plantaciones de leucaena cubriendo un área total de más de 18,400 ha. Una sólida red de investigación y desarrollo abordó temas como el tratamiento de la semilla, su germinación, la inoculación con rizobios, la configuración de las plantaciones, los modelos agroforestales, la selección y el desarrollo de clones de alta producción, el establecimiento de bancos clonales para la producción de semillas, el mejoramiento genético mediante técnicas de mutación, y programas de hibridación para mejorar la calidad de la madera.

Palabras clave: Agroforestería, fitomejoramiento, hibridación, madera para pulpa de papel, mutación, plantaciones.

Introduction

JK Paper Ltd has an annual production capacity of 550,000 t/yr with 3 integrated pulp and paper plants located at Songadh (Gujarat), Rayagada (Orissa) and Kagaznagar (Telangana) producing writing and printing paper and virgin packaging boards. JK Paper Limited, Central Pulp Mills (CPM) Unit, is the largest integrated pulp producer in Gujarat with a paper and paperboard manufacturing unit located at Fort Songadh, Tapi District, Gujarat State, India, producing 155,000 t paper and paperboards annually. The annual wood requirement of CPM unit is about 275,000 t comprising primarily *Leucaena*, *Eucalyptus* and *Casuarina*, of which *Leucaena* is the major contributor (about 75%). To achieve a sustainable raw material supply, JK Paper Ltd has promoted social and farm forestry plantation programs in the mill's catchment area since 1996-1997. CPM unit provides quality seeds and improved clones at subsidized prices and provides free technical support to the farmers, including a guaranteed market for their harvested wood.

Plantation research and development and operational procedures

JK Paper Ltd, CPM unit, was originally based on using bamboo as raw material from leased forest areas from 1960 to 2006. During 2006, gregarious flowering in bamboo forests took place in south Gujarat forest areas, following which many bamboos died and productivity of bamboo was reduced from 100,000 to 20,000 t/yr. This led to a social and farm forestry plantation program promoting *Eucalyptus*, *Casuarina* and *Leucaena* species. A massive promotional drive to establish leucaena plantations was initiated from 2009 in Tapi, Surat, Navsari, Valsad, Bharuch, Narmada, Vadodara, Panchmahal, Anand, Kheda and Sabarkantha districts of Gujarat and Nandurbar, Dhule and Jalgaon districts of Maharashtra State. The program targeted mostly agricultural lands, farm bunds, arable waste areas and community lands (surplus land available with public sector units and state forest corporation lands for plantations under different agro- and farm forestry models). Initially direct sowing of seed was adopted for on-farm plantings, which was later slowly replaced by sowing of rooted seedlings of improved clones. For farm forestry, *Leucaena leucocephala* (K636 and K8 provenances) were planted and robust plantation R&D programs put in place to address issues of improving seed germination through chemical and mechanical treatment, and enhancing wood production through cloning of desired plant types. A hybridization program was initiated to enhance wood production plus disease and pest resistance. Mutation techniques were used to enhance wood production.

Following robust plantation research and development work, the CPM unit developed 40 different cultivars that were site-specific, disease-resistant and high-yielding from *Eucalyptus*, *Leucaena* and *Casuarina* species giving higher wood production (3–4 times more than from plantations planted with seed, and a shorter rotation age of 3 years). JK Paper Ltd, CPM unit, has about 18,400 ha of plantations in association with >7,800 farmers in Gujarat and Maharashtra States. CPM unit on-farm procedures are illustrated in Figures 1 and 2.

Figure 1a. Land preparation for leucaena plantations, showing drip irrigation lines.

Figure 1b. Sowing seed and using drip irrigation methods.

Figure 1c. Seed germination.

Figure 1d. Mechanized inter-row cultivation.

Figure 1e. Manual inter-row cultivation.

Figure 1f. 2.5-year-old mature leucaena plantations.

Figure 2a. Leucaena cuttings propagated in misting chambers.

Figure 2b. Two-year-old leucaena plantation at Surat.

Figure 2c. Leucaena with cotton intercropping.

Figure 2d. Leucaena with banana intercropping.

Extension and motivational efforts

A strong extension network involving local influential persons plus non-government (NGO) and Government agencies was established. Farmer meetings were organized to develop awareness among the farmers regarding the economic benefits available from pulpwood plantations. To instill confidence in this system we organized exposure visits to successful plantations in Andhra Pradesh, where an extensive area was covered with leucaena plantations, and to our mill and R&D Centre. Promotional stalls were established at different agricultural exhibitions in Gujarat and Maharashtra giving demonstrations regarding the economic and environmental benefits of plantations (Table 1).

Site preparation

Leucaena plantations were established within a 0–350 km radius of the mills in Gujarat and Maharashtra States. The majority of soils are black alkaline soils formed from basalt rock and the major agricultural crops are cotton, sugarcane, banana, papaya, black gram, green gram and *Cajanus cajan*. Deep ploughing with a mould-board plough followed by harrowing was recommended and organic manures such as cow dung were added prior to sowing. Most plantations are under a drip irrigation system.

Establishment procedures

Most farmers adopted tree spacings from the following range: 1.2 × 1.2 m, 1.5 × 1.0 m, 1.5 × 1.2 m and 1.0 × 3.0 m, which allowed intercropping in the first year. Seed was treated to ensure uniform and fast germination (70–80%), a critical factor in establishment of leucaena plantations. Methods for breaking leucaena seed dormancy included chemical treatment (99% H_2SO_4) and mechanical scarification with a Kimseed seed scarifier imported from Australia.

Seed is sown at the onset of the monsoon, i.e. in June-July, with 2 or 3 seeds per hole and irrigation is available at the time of sowing. Following germination, weeding is performed and leucaena plants thinned to 1 healthy seedling per location when they reach a height of 15–20 cm.

Table 1. Numbers of farmer meetings, farmer tours and visits to agricultural exhibitions.

Year	Farmer meetings at CPM		Farmer tours to Andhra Pradesh		Agricultural exhibitions	
	No. of meetings	No. of farmers	No. of tours	No. of farmers	Total	Total farmers (00,000)
2009/10	13	720	5	135		
2010/11	28	1,310	2	34		
2011/12	20	470			2	2.2
2012/13	78	715			3	0.1
2013/14	165	831			2	0.1
2014/15	129	305			2	0.1
2015/16	99	865			2	0.2
2016/17	57	570			1	0.1
2017/18	80	800			5	0.5
Total	669	6,586	7	169	17	3.3

The establishment cost of leucaena plantations is US$ 688/ha.

Rhizobia inoculation

Establishment of the rhizobium association proved to be sporadic. In order to enable faster growth, rhizobium cultures/colonies from different areas were tested with the help of M/s PAC Bio Fungbact Pvt Ltd, Madhi, Gujarat. It was found that a mixture of all rhizobia was best for helping development of profuse nodulation in seedlings (Figure 3). With the help of M/s PAC Fungbact Pvt Ltd, a rhizobium culture was developed in powder form and is being supplied to farmers along with seeds, where it is coated on seeds prior to sowing.

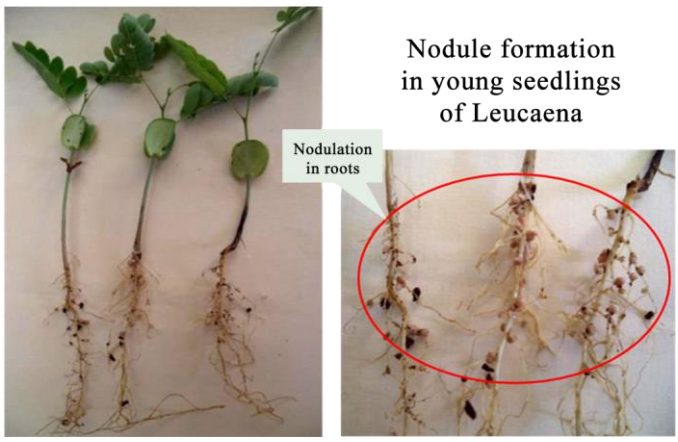

Figure 3. Rhizobial nodulation in leucaena.

Protection and maintenance of plantations

Where irrigation is available, it is applied every 15–30 days depending on soil moisture conditions. Fertilizer applications are based on soil testing and comprise 50 g NPK (12:32:16)/plant on 2 or 3 occasions for the first 2 years. In potash-deficient soils, an additional 50 g potash/plant is applied. Application of good quality organic manure is recommended, but no grazing, browsing or trampling is allowed. Termite damage is controlled by applications of 0.05–0.10% chlorpyriphos (5–10 mL/L of water) to soil around the base of the plant.

Agroforestry intercropping with leucaena

Intercropping of leucaena plantations in the first year is often practiced with cotton, ground nut, pigeon pea, green gram, bananas, onions, pigeon pea, chilli, castor oil, sugarcane or ginger. Farmers find that leucaena has no adverse effects on crop production in the first year. They report a range of benefits of intercropping, including:

- Higher returns/profits in comparison with normal agricultural crops, and reduced risk of crop failure;
- Nitrogen fixation by leucaena as a leguminous plant;
- Fodder for cattle feed;
- Fuel wood;
- Soil fertility improvement due to germination of fallen seeds that become bio-fertilizers;
- Humus formation by continuous fall of dead leaves;
- Pulpwood generation; and
- Environmental benefits due to carbon storage, reduced soil erosion and improved soil moisture retention.

Leucaena research and development

Productivity improvement through Candidate Plus Trees [CPTs]

In order to have a broader genetic base and to improve yield per unit area, a systemic genetic approach in research and development of leucaena is being under-taken. Selection of CPTs in Gujarat and Maharashtra

States at different sites is in progress. To date, about 1,300 CPTs have been selected. A further short list of the top 10 CPTs was selected for testing of pulping properties at our R&D laboratory. Screened pulp yields (pulp/fiber % of wood) varied from 47.2 to 51.4% (Table 2), slightly higher than the present average screened pulp yield of commercialized leucaena clones (about 47%). We have also collected coppice cuttings from these CPTs and have developed rooting methodologies in misting chambers.

Progeny testing for these CPTs is on-going. Initial results show that CPM 3, CPM 29 and CPM 32 have 125% growth compared with the control of existing leucaena field clonal plantations. Vegetative multiplication is on-going for further multi-location trials. Presently we are producing 6 leucaena clones and many more are in the pipeline to be released shortly.

Hybridization program in leucaena

In the first phase of this program, potential species used for crossing to produce hybrid vigor and higher pulp yield and wood production were *Leucaena collinsii* and *L. leucocephala* (CPM 11 and CPM 16 clones).

Leucaena collinsii, which is diploid (2n) with 52 chromosomes, was fast-growing and resistant to psyllids (*Heteropsylla cubana*) and grew up to 8–10 m in height in 2.5–3 years. It also produced less seed, resulting in faster vegetative growth.

The clones of *L. leucocephala*, which is a tetraploid (4n) with 104 chromosomes (Brewbaker 1988), were also fast-growing and grew up to 10–12 m in height in 2.5–3 years but were susceptible to the psyllid insect, resulting in loss of growth for 8–9 months in a 3-year rotation cycle. This species produced abundant seeds, resulting in less vegetative growth during seeding. *L. leucocephala* (CPM 16) was used as a male parent and *L. collinsii* as the female parent. About 100 flowers were emasculated for crossing and observed for maturity of their stigmas. The calyx was sprayed with IAA to avoid abscission of flowers during hybridization (Sorensson 1988), and all remaining flowers were removed. We produced 15 pods through this hybridization and subsequently grew seedlings in plastic containers from the seeds (Figure 4). A hybridization test carried out at JK Agrigenetics Ltd, Hyderabad confirmed that they were true hybrids (Figure 5). We planted progeny trials to study growth of the hybrids in the field. As *L. leucocephala* is tetraploid and *L. collinsii* is diploid, the hybrid is triploid and fully-sterile and hence must be multiplied from rooted cuttings. This work is on-going.

Table 2. Pulp quality, chemical consumption, pulp viscosity, pulp brightness, cooking condition for pulp yield for leucaena wood samples collected from 6 Candidate Plus Trees (CPTs).

Parameter	Clone/CPT											
	CPT 54		CPT 3		CPT 42		CPT 29		CPT 30		CPT 32	
Age of CPT (years)	3		3		3		1.5		1.5		1.25	
Cooking chemical[1] for pulping (AA) as Na_2O (%)	19	20	19	20	19	20	19	20	19	20	19	20
Pulping results												
Kappa no.[2]	17.5	16.9	17.3	16.6	16.5	15.6	16.9	16.4	16.0	15.5	15.9	15.3
Total pulp yield (% of BDMT[3] wood)	51.3	50.9	48.4	48.1	52.1	51.8	50.6	49.8	51.1	49.7	51.2	50.9
Reject (% of BDMT wood)	1.02	0.92	1.00	0.89	0.72	0.53	0.98	0.64	0.83	0.65	0.90	0.85
Screened pulp yield (% of BDMT wood)	50.3	50.0	47.4	47.2	51.4	51.3	49.6	49.2	50.3	49.0	50.3	50.0
Free alkali as Na_2O (g/L)	9.3	9.9	12.4	13.6	11.8	13.6	10.5	11.2	10.5	11.2	10.5	11.8
Brightness (%)	28.5	29.6	29.2	30.7	30.3	32.1	31.6	32.4	32.4	33.0	32.0	32.6
Viscosity (Cps)	16.5	15.3	16.8	15.0	16.2	15.2	16.8	15.0	16.3	15.0	16.6	15.1

[1]Cooking chemical for pulping (AA) as Na_2O (%) refers to % of white liquor required for cooking/pulping of wood chips in digester.
[2]Kappa number: an indication of the residual lignin content or bleachability of wood pulp by a standardized analysis method.
[3]BDMT = Bone dry metric tonne (= at 0% moisture).

Figure 4. Breeding of *L. leucocephala* and *L. collinsii.*

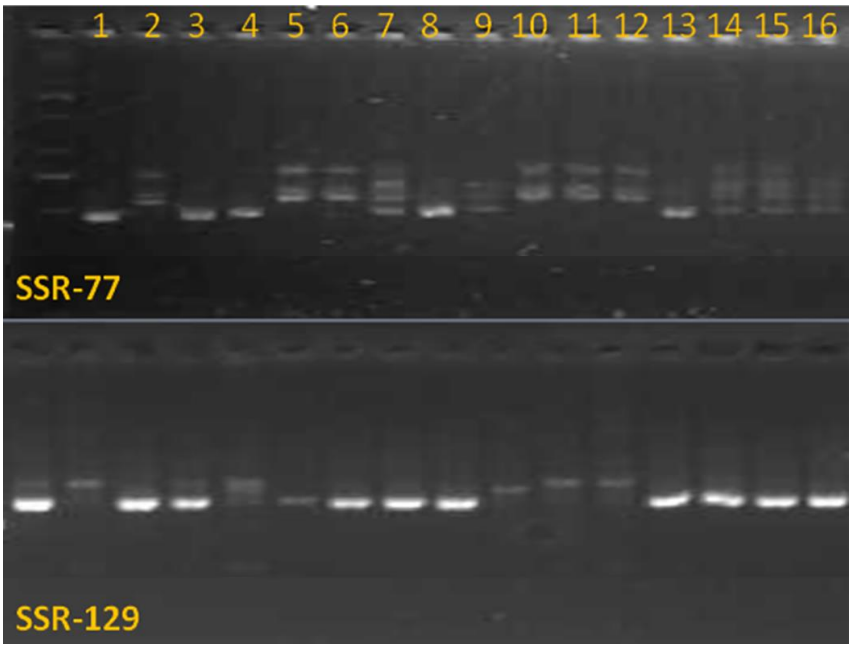

Figure 5. Leucaena samples- 1: K636 (mutated); 2: K636; 3: CPM 11 (mutated); 4: CPM 11; 5: CPM 16 (mutated); 6: *L. collinsii*; 7: CPM 16; 8: *L. collinsii* (mutated); 9: KX2; 10: CPT 32; 11: CPT 3; 12: CPT 29; 13: Hy 1; 14: Hy 2; 15: Hy 3; 16: Hy 4. Status of sample 6 (*L. collinsii*) - diploid; sample 7 (CPM 16) - tetraploid; samples 14, 15 and 16 - triploid – was confirmed as true hybrids by JK Agrigenetics.

Mutation techniques in leucaena

We used gamma ray mutation techniques for alteration of gene structure, which may transmit to coming generations. We irradiated leucaena seedlings of K636 (known as cv. Tarramba in Australia), *L. collinsii*, *L. leucocephala* clone CPM 11 and *L. leucocephala* clone CPM 16 with different frequencies of gamma rays at the nuclear research station, Indian Agriculture Research Institute, New Delhi and laid out progeny trials in August 2016 for studying the effects of mutations.

Based on the superior induced growth in some mutants, DNA fingerprinting analysis was carried out, which confirmed that mutations altered the gene structure and growth has been accelerated. Vegetative multiplication of positive mutants is on-going for further trials.

Clonal hedge garden techniques

A naturally ventilated polyhouse covered with 200 micron polythene stabilized against UV rays and provided with a fertigation system, plus temperature and humidity controllers, was constructed. Superior mother plants were planted in raised beds filled with pure sterilized sand at 10 × 10 cm spacing. Required fertilizer dosages were provided to the plants through the fertigation system and constant humidity and temperature were maintained. Every month about 3 or 4 juvenile coppice cuttings were obtained from each mother plant. The adequate nutritional status of the mother plants was important in increasing the rooting percentage of cuttings in the misting chamber.

Propagation techniques

Cocopeat was used for clonal propagation as it has low salinity as measured by electrical conductivity (EC). It also has excellent water holding capacity and cation exchange capacity. Fifty mL, 60 cell plastic root trainer blocks were used for production of clones (Lal 2001). Misting chambers with appropriate temperature and humidity control systems were installed over a 3,200 m^2 area at the clonal propagation centre in Songadh. Every month about 150,000 juvenile apical cuttings are established producing 1 million leucaena clones per annum. Water quality is critical for a successful misting chamber operation. Water used has a pH of 6.5–7.5, very low EC and sodium absorption ratio below 3. Reverse osmosis water rather than canal or river water is preferable for misting chamber operation (Brewbaker 1988).

Outcomes and discussion

Increased plantings

To date the CPM Unit has promoted the establishment of approximately 50,000 ha of social and farm forestry plantations in Gujarat and Maharashtra States (Figure 6) involving about 66,000 farmers. Similarly, 18,400 ha of leucaena plantations have been established by approximately 7,800 farmers (Figure 7).

Wood asset value of plantations

Wood generated from plantations promoted by JK Paper Ltd is being used for making paper, plywood, poles and furniture. The expected annual increase in value of wood is given in Figure 8. JK Paper Ltd, CPM unit plantation initiatives are creating sustainable livelihoods among nearby farmers by creating economical wood assets on their farm lands.

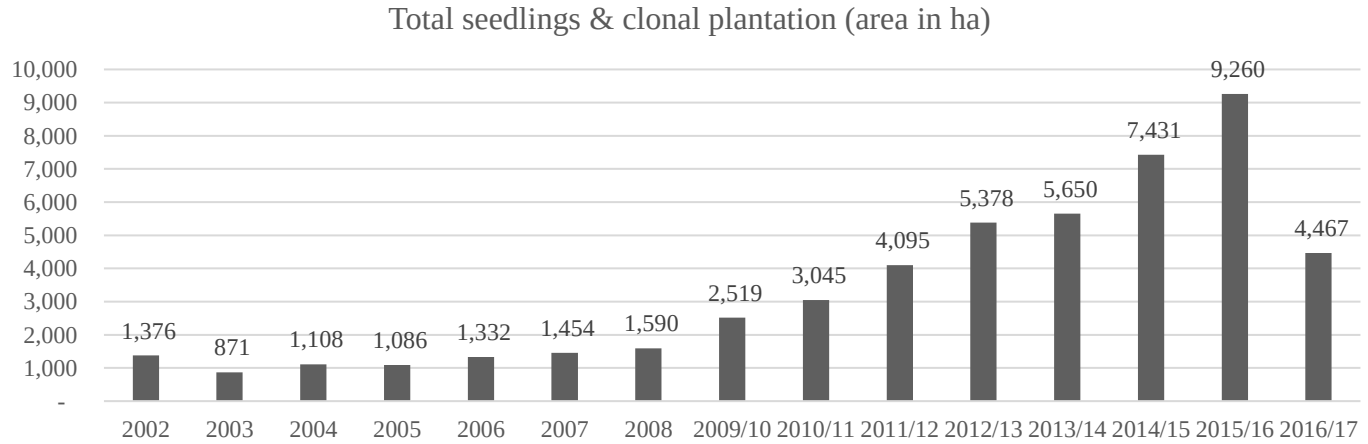

Figure 6. Annual increases in area under seedling and clonal plantations (ha). Note: The area planted in the year 2016/17 was low due to higher wood availability and lower wood requirement.

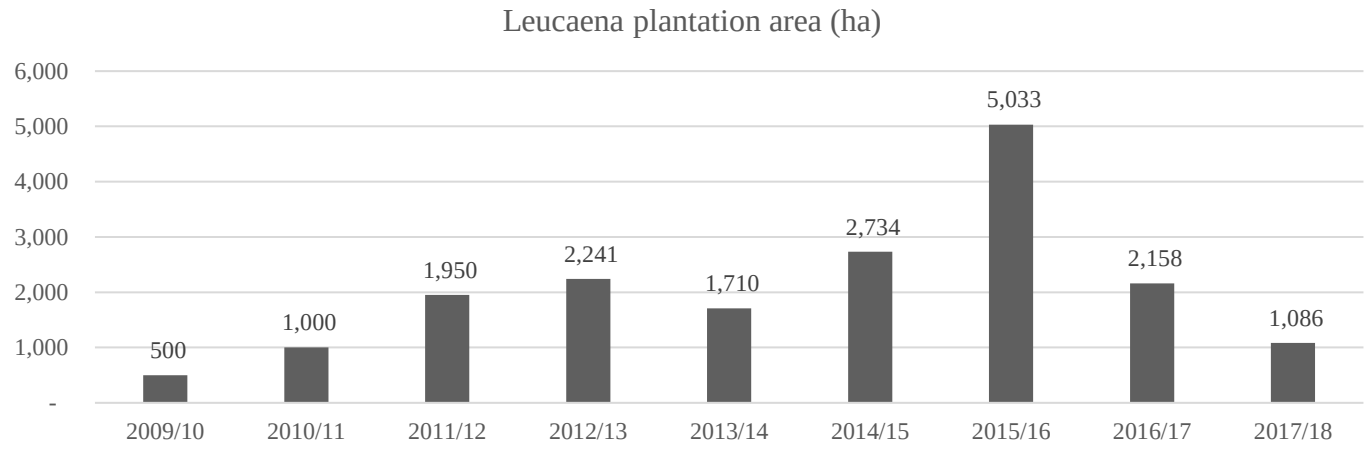

Figure 7. Annual increases in area under leucaena plantations (ha).

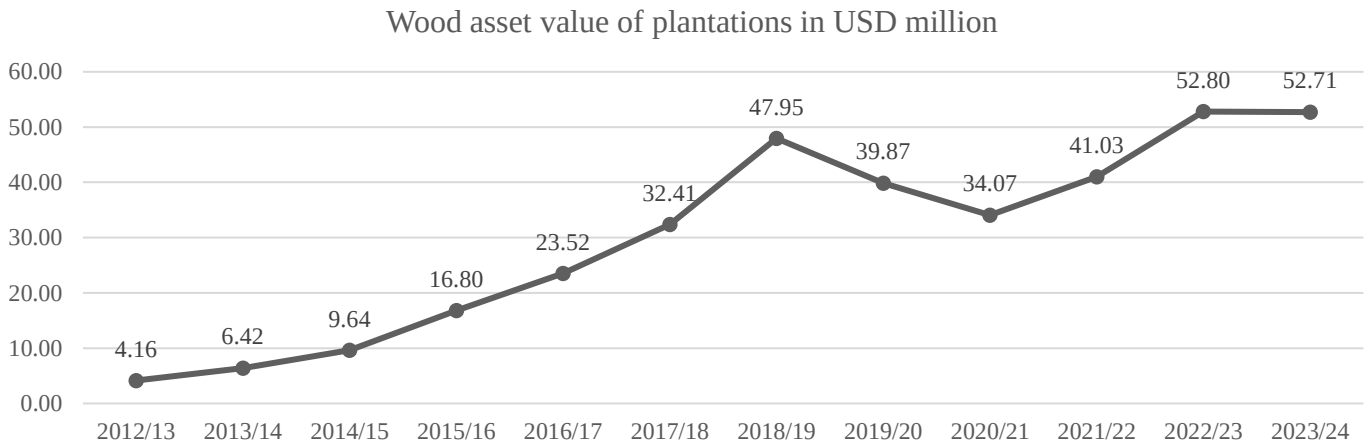

Figure 8. Estimated and projected wood asset value of plantations in millions of USD.

Survival, productivity and environment

Hi-tech clonal plantations in areas surrounding the mill have >90% survival. With continuous research and development efforts, site-specific, disease-resistant, fast-growing and high-yielding clones achieved a productivity of 30–50 t/ha/yr.

The value of JK Paper Ltd farm forestry program is immense in mitigating environmental degradation. Apart from increasing greenery and tree cover, farm forestry has significant potential for carbon storage. Estimated quantities of CO_2 extracted from the air and C stored by farm forestry during the period 2012/13 to 2016/17 are shown in Table 3.

Table 3. Estimated annual carbon storage and CO_2 absorption by standing plantations.

Sl No.	Year	Plantation area (ha)	Wood production of farm forestry (t)	Carbon stored [t]	Carbon dioxide absorbed [t]
1	2012/13	5,378	239,847	119,924	439,720
2	2013/14	5,650	350,864	175,432	643,251
3	2014/15	7,431	483,433	241,717	886294
4	2015/16	9,260	770,842	385,421	1,413,210
5	2016/17	4,467	593,615	296,808	1,088,294

Conclusions

Leucaena clonal programs have taken 'deep roots' among the farmers in Gujarat and Maharashtra States. This has increased wood production per unit area by 3–4 times compared with seed-planted plantations, thereby increasing net economic returns to the farmers. Clonal *Eucalyptus*, *Casuarina* and *Leucaena* plantations are making immense contributions towards development of wood-based industries, local asset value addition, employment generation, diversification of agriculture, greening of the country and environmental amelioration. Likewise, clonal technology, supported with an improved package of silvocultural management techniques and due safeguards, offers opportunities for substantial improvements in production of plantations and significant enhancement of quality of plantation-grown timber.

Establishment of about 50,000 ha of plantations involving 66,000 farmers in areas surrounding the JK Paper Ltd, CPM unit mill has created a viable and sustainable economic model for farmers, transporters, paper mills and laborers. With these plantations, the CPM unit has developed a sustainable fiber resource to cater for raw material needs into the future. While substantial advances have been made, much more needs to be done to increase productivity and to improve quality of the end products to match international standards.

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ILC2018 Poster and Producer paper*

Genetic improvement of *Leucaena leucocephala* for wood energy

Mejoramiento genético de Leucaena leucocephala *para bioenergía*

RINA LAKSMI HENDRATI AND SITI HUSNA NURROHMAH

Centre for Forest Biotechnology and Tree Improvement Research and Development, Indonesian Ministry of Environment and Forestry, Yogyakarta, Indonesia. menlhk.go.id

Keywords: Bioenergy, biofuels, tree legumes.

Introduction

The world demand for woody biomass for energy generation is increasing rapidly (Rakos 2008; Spelter and Toth 2009; Sikkema et al. 2011). Woody biomass from short-rotation crops can contribute to secure renewable and sustainable energy around the world owing to their potential to produce high biomass in short time periods, especially in tropical countries with plentiful rain (Hendrati 2016). Many fast-growing species have high wood quality for energy and an ability to re-sprout for multiple harvests, which is important for economic success. Multipurpose species provide multiple environmental and rural development benefits (Singh et al. 2010) and with genetic improvement, further improvement in yield and efficiency of production are anticipated. Studies on genetic improvement of *Calliandra calothyrsus* for wood energy indicated high heritability value of wood volume ($h^2 = 0.5$) and an increased yield of 75% for wood volume (Hendrati 2016). This paper describes research on the genetic improvement for wood energy of *Leucaena leucocephala,* which is self-fertile and therefore less variable than *Calliandra calothyrsus*, which is not self-fertile. Nevertheless, as 2 subspecies of *Leucaena leucocephala* (ssp. *glabrata* and *leucocephala*) are present in Indonesia, there is some ability for outcrossing, so genetic gain is achievable.

Materials and Methods

Genetic improvement of *L. leucocephala* ssp. *glabrata* (imported leucaena) was initiated by collecting genetic material from 10 established orchards, including cv. Tarramba, during the period 2015–2016. They were from Subang and Majalengka (West Java), Brebes (Central Java), Sleman, Bantul and Kulon Progo (DIY), Bangkalan Madura (East Java), Bali (Bali Island), Menado (North Sulawesi) and Kupang (East Nusa Tenggara). At most sites, the species was grown by villagers for forage, fuelwood and human consumption (seed). *Leucaena leucocephala* ssp. *glabrata* was preferred over common local leucaena (*L. leucocephala* ssp. *leucocephala*) because it has better growth and is more tolerant of psyllids (*Heteropsylla cubana*) than the more susceptible ssp. *leucocephala*. The range in elevations from which these samples were collected was 0–500 masl and precipitation ranged from 800 to 3,050 mm/yr. Open-pollinated half-sib seeds from 80 trees (considered as families hereafter), selected as the best performers in the orchards, were collected. *Leucaena leucocephala* is considered to be a cross-pollinating species but up to 10% selfing is known to occur. Consequently, the collected seeds were considered F1, although some seed may have resulted from self-pollination. A long-term breeding strategy was planned as shown in Figure 1. Progeny tests were established at 2 locations, Wonogiri and Brebes, Central Java (Table 1). This phase of the program is represented by the box 'Progeny test F1 on 2 sites'. Distance between individual mother trees (families) within each population was 70–100 m to avoid inbreeding. Seedlings in the nursery were measured for both stem diameter and height after 4 months and again 6 months after transplanting into the field. Biomass yield after 6 months in the field was estimated using a biomass index (BI; basal diameter^2 × height; Stewart and Salazar 1992). Data from the nursery and from the field were analyzed by using analysis of variance and Duncan's Multiple Range Test.

Correspondence: R.L. Hendrati, Jl. Palagan T. Pelajar km 15, Purwobinangun, Pakem, Sleman, Yogyakarta 55582, Indonesia. Email: rina.l.hendrati@biotifor.or.id

*Poster presented at the International Leucaena Conference, 1–3 November 2018, Brisbane, Queensland, Australia.

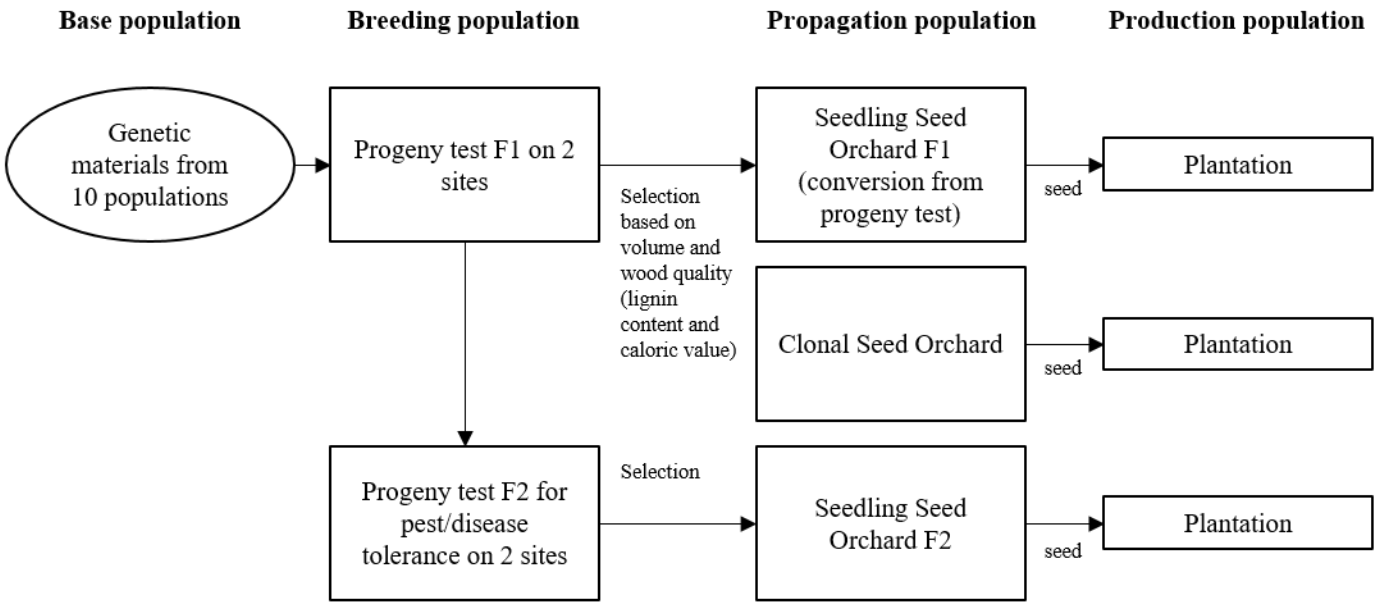

Figure 1. Breeding strategy for *L. leucocephala* to increase wood energy production.

Table 1. Description of *L. leucocephala* ssp. *glabrata* progeny tests established at 2 sites.

No.	Location	No. families	No. populations	Spacing/ block/design	Individuals per family/block	Precipitation (mm/yr), soil type
1	Ketanggungan, Brebes, Central Java	80	10	2 × 2 m/6 blocks/single tree plots	4 (Total = 1,920)	1,961, Vertosol
2	Girimulya, Wonogiri, Central Java	80	10	2 × 2 m/8 blocks/single tree plots	4 (Total = 2,560)	1,800, Alluvial

Early results

After 4 months of growth in the nursery, seedlings were ready for transplanting. At this time, variations in both height and stem diameter were obvious (Table 2). Bantul, Bali, Menado and Kulon Progo populations had diameters comparable with that of cv. Tarramba; for height, only the Bali population was similar to cv. Tarramba. The Subang population always recorded the lowest values for both characters.

Variations between families were re-examined after 6 months in the field (Table 3). While there were significant differences between the 80 families for stem diameter, height and biomass yield (Table 3), results for the best 8 performers for each parameter were not significantly different (P>0.05) (Table 4). Tarramba did not fall within this group for stem diameter but 3 Tarramba families fell in the top 8 families for both height and biomass yield (Table 4).

Table 2. Analysis of growth (diameter and height) of 10 *L. leucocephala* populations from Indonesia after 4 months in the nursery.

Origin	Diameter (cm)	Origin	Height (cm)
Kupang (cv. Tarramba)	0.310a[1]	Kupang (cv. Tarramba)	35.68a
Bantul	0.305a	Bali	35.01a
Bali	0.299ab	Majalengka	32.99b
Menado	0.294ab	Sleman	32.73b
Kulon Progo	0.294ab	Brebes	32.55b
Sleman	0.281bc	Bantul	32.48b
Madura	0.275c	Menado	32.43b
Brebes	0.275c	Madura	31.28b
Majalengka	0.275c	Kulon Progo	31.15b
Subang	0.256d	Subang	27.85c

[1]Means followed by the same letter are not significantly different (P>0.01) by Duncan's Multiple Range test. Source: Hendrati and Hidayati (2018).

Table 3. Results of statistical analysis for growth (diameter, height and biomass) of 80 *L. leucocephala* families after 6 months in the field.

Trait	SV	df	SS	MS	P>F
Diameter	Fam	79	102842.5	1301.8	0.004 ***
Height	Fam	79	650168.9	8229.9	<0.001 ***
Biomass	Fam	79	30555694.6	386780.9	0.003 ***

Table 4. The best 8 of the 80 *L. leucocephala* families tested for diameter, height and biomass after 6 months in the field.

Rank	Population	Best 10% (family)	Value
Diameter (mm)			
1	Bali	40	69.5
2	Majalengka	27	58.6
3	Majalengka	17	56.8
4	Bali	39	55
5	Subang	3	54
6	Subang	9	53
7	Brebes	34	53
8	Bantul	73	52
Height (m)			
1	Kupang (cv. Tarramba)	52	2.38
2	Kupang (cv. Tarramba)	51	2.34
3	Brebes	32	2.29
4	Majalengka	27	2.26
5	Menado	45	2.25
6	Majalengka	20	2.22
7	Kupang (cv. Tarramba)	57	2.19
8	Bali	40	2.18
Biomass index (d^2×ht)			
1	Kupang (cv. Tarramba)	51	1,000
2	Kupang (cv. Tarramba)	52	964
3	Menado	45	851
4	Majalengka	20	729
5	Brebes	37	727
6	Kupang (cv. Tarramba)	57	709
7	Brebes	32	686
8	Majalengka	15	665

Family correlations (n = 80) between growth in the nursery and in the field were significant only for height (y = 0.05 x + 22.873; r = 0.308**).

Discussion

Environmental factors were relatively uniform in the nursery and in the field. Therefore, growth was assumed to be influenced more by genetic potential than by environmental conditions. Variations in terms of growth (diameter and height) both in the nursery and in the field were expected to optimize selection to achieve genetic gain during the improvement program. While some families showed promise in terms of diameter and others were outstanding in terms of height, wood biomass as indicated by the biomass index was most important and families, which scored well in this parameter, are of most interest. Some Indonesian populations and families were comparable with those from cv. Tarramba, which is known for its superior growth compared with other cultivars (Rengsirikul et al. 2011).

Significant correlations between heights of families in the nursery and in the field indicated that good height in the nursery might indicate good height in the field. Families with high ratings for biomass production will be evaluated in terms of wood volume and quality for energy at the appropriate age to supplement current growth assessments. Outstanding families will progress through the breeding program.

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