Off-types indicate natural outcrossing in five tropical forage legumes in Colombia

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Abstract

Flower colour has been used as a simple morphological marker for genetic studies in many species, including legumes. In several tropical forage legumes, grown to evaluate their environmental adaptation or for seed increase, white-flowered plants were observed when the predominant flower colour was either yellow (*Chamaecrista rotundifolia*) or pink, lilac or purple (*Centrosema virginianum*, *Codariocalyx gyroides*, *Desmodium heterocarpon* and *Galactia striata*). Open pollination of these 5 species took place each at one of 4 sites in Colombia. Progeny from white-flowered plants were examined for flower colour to assess the proportion of off-types. This ranged on average from 4% for *D. heterocarpon*, through 13% for both *G. striata* and *Ch. rotundifolia*, and 18% for *Ce. virginianum*, to 23% for *Co. gyroides*. Large differences were recorded among accessions of the same species, particularly in *Ce. virginianum*, where 4 of the accessions apparently produced autogamous offspring and the other 5 had high proportions of off-types, with a rate as high as 89%. Consequences of these results for germplasm collection, management and seed increase are discussed.

Introduction

The rate of outcrossing under conditions of open pollination is important, both for germplasm acquisition and preservation of the genetic integrity of an accession or released cultivar. It

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also influences the regeneration protocol in a germplasm bank and how large-scale seed production of commercial materials is managed, and has relevance for designing efficient plant breeding programs.

Little is known about the reproductive biology of many of the tropical legume species used for pasture or soil improvement. Hutton (1960), who studied flowering and pollination in some tropical pasture legumes, stated that most were cleistogamous, *i.e.* pollinated within the closed bud. In contrast, *Desmodium uncinatum* and *D. sandwicense* were mainly cross-pollinated. However, the rate of outcrossing differs among species and among accessions or cultivars of the same species according to environmental conditions, as in pigeon pea (*Cajanus cajan*) (Saxena *et al.* 1994), and also pollinator preferences, as in alfalfa (*Medicago sativa*) (Steiner *et al.* 1992). To determine the rate of outcrossing, a genetic marker needs to be established, whether morphological, biochemical or molecular. A simple genetic marker may be preferred because of cost.

Together with other morphological markers, flower colour has been used traditionally for genetic studies. Many tropical forage legumes contain white-flowered plants which, in some cases, have been separated as distinct botanical forms, *e.g. Desmodium heterocarpon* subsp. *heterocarpon* var. *heterocarpon* f. *albiflorum* (Ridley) (Ohashi 1991).

A model of simple inheritance of absence (= white flowers) or presence of colour has been reported in various legume species, for example in *G. max* (Hartwig and Hinson 1962), *Stylosanthes guianensis* (Miles 1985), *D. heterocarpon* (Quesenberry *et al.* 1989), *M. sativa* (Talbert and Bingham 1989), *Centrosema brasilianum* (Maass and Torres 1993) and *Clitoria ternatea* (J.W. Miles, personal communication). Using white flower colour as a recessive marker, Miles (1985) demonstrated an outcrossing rate of 6.7% in 12 single-plant, open-

pollinated progenies of *S. guianensis*. When a white-flowered line of *Cl. ternatea* was interplanted with a blue-flowered line at Palmira, Colombia, almost 23% of outcrossing was found (J.W. Miles, personal communication). At the same site, Maass and Torres (1992) determined an outcrossing rate ranging from 31.2–53.5% in open-pollinated *Ce. brasilianum*, showing that the white flower colour was recessive and of simple inheritance (Maass and Torres 1993). As a recessive marker, the white flower colour has thus provided a simple tool for estimating the outcrossing rate in various legumes. It was therefore used to assess the proportion of coloured-flowered off-types in the progeny of open-pollinated, white-flowered germplasm accessions of 1 tropical Caesalpinoideae and 4 Papilionoideae species, assuming that white phenotype is the result of homozygous recessive genotype under single gene control and will breed true for this character on selfing.

Materials

The species and selection of genotypes

In several species, initially grown to evaluate their environmental adaptation or for seed increase, germplasm accessions with white-flowered plants were observed when the predominant flower colour was either yellow (*Chamaecrista rotundifolia*) or pink, lilac or purple (*Centrosema virginianum*, *Codariocalyx gyroides*, *D. heterocarpon* and *Galactia striata*).

The pasture legume *Ch. rotundifolia* (Caesalpinoideae:Cassieae) occurs widely throughout tropical America (Irwin and Barneby 1982). In a germplasm collection of 77 accessions of *Ch. rotundifolia*, planted randomly in 1993 (10 plants per plot, planted in rows 3 m apart, 3 replications) and evaluated for environmental adaptation at Carimagua in the Colombian Eastern Plains, little morphological variation was observed within accessions. One accession from Monagas, Venezuela, had both white- and yellow-flowered plants. While describing the species' morphology to be highly variable, Irwin and Barneby (1982) did not mention any petal colour other than yellow, drying pinkish- or orange-brown. *Ch. rotundifolia* was a heavy seed producer at Carimagua even though insect visitation was infrequent (B.L. Maass and E.A. Cárdenas, unpublished data).

Centrosema virginianum (Papilionoideae: Phaseoleae) is the most widely occurring species of *Centrosema*, being found from 35°S to 40°N in the Americas. Its agronomic potential has been identified particularly for subtropical and dry environments and for high-altitude tropics (Schultze-Kraft *et al.* 1990). In a 1989 field multiplication plot (5 plants per accession, planted randomly and unreplicated in rows 2 m apart) at CIAT close to Palmira, Colombia, 9 of 198 accessions were uniformly white-flowered. Three of these accessions originated from Bolívar, Venezuela, and the remaining 6 from the Brazilian states of Bahia, Espírito Santo and São Paulo. Insect visitation, particularly of bumblebees, was frequent (A. M. Torres, unpublished data).

Galactia striata (Papilionoideae:Phaseoleae) is of tropical American origin. It is especially appreciated for its high forage value during the dry season (Vera *et al.* 1983). A large germplasm collection of *G. striata* was established in a randomised block design (10 plants per plot, planted in rows 3 m apart, 2 replications) at Carimagua in 1993, to assess genetic diversity and environmental adaptation. Although little morphological variation was observed within accessions, individual plants within the same accession showed large differences in vigour. Six of 379 accessions were uniformly white-flowered: 1 from Bahia, Brazil; 1 from Honduras; and the remaining 4 from the hinterland of Santa Marta in the Colombian North Coast. Some insect visitation was observed in *G. striata*, but most flowers were aborted and little seed was harvested, probably because of low soil fertility (B.L. Maass and E.A. Cárdenas, unpublished data).

Desmodium heterocarpon (Papilionoideae: Desmodieae) has been developed as a pasture legume that withstands heavy grazing (Hacker and Kretschmer 1992). For germplasm characterisation and preliminary evaluation of this southeast Asian species, a trial was planted at Santander de Quilichao, Colombia, in 1989 (5 plants per plot, 3 replications). Of 213 accessions, 6 were uniformly white-flowered: 4 from Malaysia and 2 from Indonesia (CIAT, unpublished data).

The south-east Asian shrub *Codariocalyx gyroides* (Papilionoideae:Desmodieae) is of agronomic interest, both as a cover crop and forage (Maass *et al.* 1996). Twenty-seven

accessions were planted in a randomised block design (10 plants per accession, planted in rows 3 m apart, 3 replications) near Florencia, Colombia, in 1992. Two accessions, CIAT 13980 from Papua New Guinea and CIAT 23736 from Indonesia, had both coloured- and white-flowered plants, although the white corolla was not mentioned in Ohashi's (1973) revision of the species. During the experiment, frequent insect visitation was observed, particularly of wild bees. Abundant seed was produced by almost all accessions in this humid, tropical lowland environment (Maass *et al.* 1996).

Methods

As described above, open pollination of the 5 species took place each at 1 of 4 sites in Colombia, where CIAT's Tropical Forages Program and Genetic Resources Unit conduct trials (Table 1). Seed was harvested from the open-pollinated, white-flowered plants only, and the progeny were subsequently examined for flower colour. The origin of these F_1 progenies is given in Table 1, together with environmental features of the 4 sites.

Where sufficient seed was available, sample sizes were calculated with 95% confidence according to seed harvested and on the basis of published records of outcrossing rates for closely related species, 18% for *Desmodium* and *Codariocalyx* (Rotar *et al.* 1967), and 42% for *Centrosema* (Maass and Torres 1992). Confidence intervals of 95% were calculated for the results obtained.

Flower colour of *Ch. rotundifolia* and *G. striata* was examined in a screenhouse at the CIAT experiment station near Palmira, in 1996, where plants were sown in plastic containers with a soil-sand mixture. All available seed was sown for *Ch. rotundifolia* CIAT 8711, but half of the plants died before they started flowering, the

remainder being 46 plants. An accumulated total of 148 F_1 progeny, mostly from CIAT 21454, was examined for flower colour in *G. striata*.

Progenies from 9 white-flowered *Ce. virginianum* accessions were examined for flower colour in 1994, and from 6 *D. heterocarpon* accessions for flower colour and leaf variegation in the field at CIAT-Palmira in 1993. However, *D. heterocarpon* was poorly adapted to the alkaline soils of Palmira and most of the about 1500 plants died. Nevertheless, data generated at that site indicated an outcrossing rate of 7.3% (13 coloured-flowered out of total 179 plants) across accessions. In 1994, about 1500 additional F_1 plants of 6 accessions were sown in an acid soil at the CIAT experiment station Quilichao.

For *Co. gyroides*, seed was harvested from the white-flowered individuals of accession CIAT 13980 only, and 266 F_1 plants were sown in the field near Florencia in May 1994, to examine them for flower colour and days to flowering.

Results

Progenies of all 5 species showed variation for flower colour, except 4 accessions of *Ce. virginianum*. Results established a proportion of off-types, on a species basis, from less than 4% for *D. heterocarpon* to 23% for *Co. gyroides* (Table 2). From a total of 1424 *D. heterocarpon* F_1 plants, a range of 1–8 plants per accession had coloured flowers, except for CIAT 23301, which was significantly higher with 35 plants. Leaf variegation of *D. heterocarpon* occurred in all plants independent of flower colour, and thus did not serve as morphological marker in this study. This is in accordance with Ohashi (1991) for typical *D. heterocarpon*, although Quesenberry *et al.* (1989) successfully used leaf marks for hybrid identification in crosses between *D. heterocarpon* and *D. ovalifolium*.

Table 1. Location and environmental features for open pollination of five tropical legume species at 4 sites in Colombia.

Species	White-flowered accessions	Location of site			Environmental features of site		
		Place	Latitude	Longitude	Altitude	Annual rainfall	Mean annual temperature
	(No/total)		(N)	(W)	(m)	(mm)	(°C)
Ch. rotundifolia	1/77	Carimagua	4°31′	71°17′	150	2300	27
G. striata	5/379						
Ce. virginianum	9/198	Palmira	3°31′	76°20′	965	960	24
D. heterocarpon	6/213	Quilichao	2°25′	76°19′	990	1800	24

Table 1. Location and environmental features for open pollination of five tropical legume species at 4 sites in Colombia.

Species	White-flowered accessions	Location of site			Environmental features of site		
		Place	Latitude	Longitude	Altitude	Annual rainfall	Mean annual temperature
Co. gyroides	2/27	Florencia	1°26′	75°26′	200	3600	26

Table 2. Off-types in different accessions of 5 open-pollinated tropical legume species in Colombia.

Species	CIAT accession		F$_1$ plants			Off-types	
	No	Origin[1]	Total	White-flowered	Coloured-flowered	Proportion	Confidence interval
			(No)	(No)	(No)	(%)	(95%)
Ch. rotundifolia	8711	VEN	45	39	6	13	3–23
Ce. virginianum	495	VEN	100	100	0	0	—
	5212	BRA	96	95	1	1	0–3.0
	5213	BRA	98	11	87	89	83–95
	5357	BRA	100	100	0	0	—
	5494	BRA	85	76	9	11	4–17
	5497	BRA	86	65	21	24	15–34
	15437	VEN	87	87	0	0	—
	15438	VEN	100	62	38	38	29–48
	25200	BRA	97	97	0	0	—
	Accumulated		849	693	156	18	16–21
G. striata	8955	BRA	16	12	4	—[2]	—
	9942	COL	24	18	6	—[2]	—
	21454	HND	108	99	9	8	3–14
	Accumulated		148	129	19	13	8–18
D. heterocarpon	13179	MYS	241	238	3	1	0–2.6
	13183	MYS	238	234	4	2	0–3.3
	13184	MYS	240	238	2	1	0–1.9
	13189	MYS	238	237	1	0.4	0–1.2
	23301	IDN	228	193	35	15	11–20
	23303	IDN	239	231	8	3	1.0–5.6
	Accumulated		1424	1371	53	4	2.7–4.7
Co. gyroides	13980	PNG	235	181	54	23	18–28

[1]Origin from the CIAT passport database; BRA = Brazil; COL = Colombia; HND = Honduras; IDN = Indonesia; MYS = Malaysia; PNG = Papua New Guinea; VEN = Venezuela.
[2]Not calculated separately because of small sample size.

Considerable differences in the proportion of coloured-flowered plants were observed in *Ce. virginianum*, ranging from 0–38%, with CIAT 5213 being significantly higher at 89%. The progeny of 4 *Ce. virginianum* accessions were apparently autogamous. These plants were rather small with little vigour and often had small and cleistogamous flowers. In contrast, the apparent allogamous plants were vigorous, and their large, showy flowers were frequently visited by insects. In *G. striata*, a substantial proportion of about 13% coloured-flowered plants was observed across accessions, whose sample sizes were too small to detect significant differences. In *Co. gyroides*, 23% coloured-flowered off-types occurred. Days to flowering ranged widely in this species, from 83 to more than 235 days after sowing, with no statistically significant difference between individuals of different flower colour.

Discussion

No study of *Ch. rotundifolia*, *Ce. virginianum*, *G. striata* and *Co. gyroides* was undertaken to confirm that white-flowered phenotype is the result of homozygous recessive genotype under single gene control, and therefore will breed true for this character on selfing. However, there are various other legume species where this model proved true (Hartwig and Hinson 1962; Miles 1985; Quesenberry *et al.* 1989; Talbert and Bingham 1989; Maass and Torres 1993). Hence, we will use the proportion of coloured-flowered phenotype (off-types) in the progeny of white-flowered individuals as an indication for natural outcrossing rate and will call this outcrossing for ease of understanding.

It is impossible to estimate bias in the observed rates of natural outcrossing for the 5 tropical legume species because the possibility that insects pollinated from 1 white-flowered individual to another within the same plot or between different plots of the same trial was not considered. Consequently, actual outcrossing rates may be higher than those calculated from the proportion of off-types. In addition, if coloured-flowered plants in the adjacent plots were heterozygous, their recessive gene for white flower colour would increase the occurrence of white-flowered, hybrid progeny.

Different proportions of the dominant allele in the pollen pool, non-random spatial distribution of plants, or non-random insect visitation because of flower colour preferences also may have affected the observed results. When reviewing insects in their role as flower visitors and pollinators, Kevan and Baker (1983) concluded that bees visit blue, purple and mauve flowers more than other insects, while bumblebees are strongly associated with blue flowers. When foraging flowers of *Corydalis cava* (Fumariaceae), bumblebees chose the white to white route more frequently than white to red magenta (Olesen and Knudsen 1994). Steiner *et al.* (1992) observed in *M. sativa* that pollinators preferred the following flower colours: cream > white > yellow. However, location interacted with the bees' preferences for flower colour.

Irwin and Barneby (1982) suggested that, in some small-flowered races of *Ch. rotundifolia*, the flower is autogamous. Autogamy is common in colonising species of disturbed and ruderal habitats, such as *Ch. rotundifolia*. However, another colonising species *S. guianensis*, which has often been collected as conspecific with *Ch.*

rotundifolia (Pengelly *et al.* 1997), showed a substantial level of almost 7% of outcrossing (Miles 1985), which corroborates with the outcrossing rate of 13% observed for *Ch. rotundifolia* in this study.

Many papilionates achieve high levels of outcrossing through a tripping device, that delivers pollen explosively. This feature is highly characteristic of some Desmodieae (Kalin Arroyo 1981). Kalin Arroyo (1981) stated that the tripping mechanism favours allogamy but does not exclude autogamy induced by insects. The rate of almost 4% across *D. heterocarpon* accessions is considerably lower than reported for *D. sandwicense* (18%) (Rotar *et al.* 1967). However, there were large differences between *D. heterocarpon* accessions. The data support the suggestion by Quesenberry *et al.* (1989) that some outcrossing in *D. heterocarpon* probably occurs under natural conditions.

Although *Co. gyroides* presented a lower outcrossing rate than did *D. sandwicense* (Rotar *et al.* 1967), it was higher than that of *D. heterocarpon*. This may have been because of the larger, showier flowers of *Co. gyroides* and great abundance of pollinators in Caquetá.

When reviewing the genetics and breeding of *Centrosema*, Miles *et al.* (1990) concluded that, while most species are highly, but not exclusively, self-pollinating, extensive outcrossing does occur in some species. Spears (1987) concluded, in his pollination ecology study, that *Ce. virginianum* was to some degree self-compatible, but that frequent insect visitation would indicate a high outcrossing rate. Records of phenotypic variation and insect visitation in *Ce. virginianum* compiled by Miles *et al.* (1990) make the finding of apparently autogamous accessions surprising, particularly since some were collected adjacent to highly allogamous materials (*e.g.* CIAT 15437 with CIAT 15438).

The accumulating data on outcrossing shows that, at least in *Ce. acutifolium*, *Ce. brasilianum* and *Ce. pubescens*, the proportion of cross-pollination is substantial, and these species should no longer be considered as self-pollinating (Maass and Torres 1992; Penteado *et al.* 1996). Data from the present study show that also in different *Ce. virginianum* accessions a wide range of outcrossing occurs, ranging from 0–89%. Those *Ce. virginianum* accessions with almost no off-types had small, often cleistogamous flowers. This observation supports Hutton's

(1960) results, that relate the occurrence of cleistogamous flowers in some tropical legumes with self-pollination. However, it seems important to desist from generalising these findings on a species basis without considering genotypic variation and the effect of environmental factors on reproductive mode. The observation of cleistogamy in a species may not be sufficient to conclude the absence of some degree of outcrossing (Battistin 1983, cited in Miles *et al.* 1990). In some species, the occurrence of cleistogamous and chasmogamous flowers may depend on the plant's developmental stage and environmental factors (Kalin Arroyo 1981).

In *G. striata*, problems in seed production occurred in Brazil and were attributed to pollinating agents (Nogueira Couto *et al.* 1997). The outcrossing rate of 13% observed in *G. striata* at Carimagua may even underestimate the potential rate, considering that some accessions grown in the glasshouse failed to set seed (J.E. Arroyo and B.L. Maass, unpublished data). Nogueira Couto *et al.* (1997) showed that substantially higher seed yields were obtained in *G. striata* because of insect pollination.

The proportion of off-types observed in this study may undergo considerable modification in different environments. For example, pollination success in *Ce. virginianum* differed depending on the abundance and diversity of pollinators, which, in turn, were partly influenced by island or mainland situations (Spears 1987).

As more data have become available, an increasing number of tropical legume species, previously thought to be autogamous, have been found to have substantial rates of outcrossing. The main implications for future collection are that an accession should be formed not just of one or a few plants, but of populations, in which individual plants participate in gene exchange. Outcrossing may result in genetic drift in germplasm accessions and cultivars. Thus, to maintain the genetic integrity of individual accessions during germplasm management, genetic shift or drift should be avoided. The number of plants used for seed multiplication needs to be sufficient to represent the variation in the population. This will also depend on the number of plants initially collected and the breeding system of the species.

Sufficient distance or isolation between plots is an additional means to prevent outcrossing during seed increase. The minimal effective isolation distance may need to be determined specifically for each site where seed is being increased, because of the possible effect of environmental conditions on outcrossing rates. Large-scale seed production of commercial materials also needs to take into account whether a species is native or introduced and whether, therefore, the risk of contamination from natural populations exists.

Acknowledgements

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