A minimum facility method for *in vitro* collection of *Digitaria eriantha* ssp. *pentzii* and *Cynodon dactylon*

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Abstract

Simple *in vitro* techniques were developed for collecting *Digitaria eriantha* ssp. *pentzii* and *Cynodon dactylon* to obviate deterioration, quarantine and bulk problems of conventional methods for collecting vegetative material. Various antimicrobial agents were tested for effective surface-sterilization of plant material and for supplementing the growth medium to reduce microbial contamination. Halazon tablets were used for surface-sterilization and a combination of Rifamycine and Benlate was the most effective medium supplement. This technique was found useful for the field collection of vegetative germplasm of the 2 species. In field trials, 75% of *D. eriantha* spp. *pentzii* and *C. dactylon* cultures were aseptic and growing after 4 weeks in culture. The cultures were transferred to natural conditions in soil and approximately 70% of the 2 species were recovered as potted plants. In a field trial with *C. dactylon*, 92% of the cultures which had formed roots and 80% of the cultures which had no roots at the time of transfer to soil were successfully established as potted plants.

Resumen

Se desarrollaron métodos simples para la colecta de Digitaria eriantha *ssp.* pentzii *y* Cynodon dactylon *con el fín de obviar los problemas típicos de los métodos convencionales para la colecta de material vegetal, tales como cuarentena, deterioro y volumen del material colectado. Se probaron varios agentes anti-microbianos por su efectividad para esterilizar la superficie del material de las plantas y para suplementar el medio de crecimiento y reducir la contaminación microbiana. Se usaron pastillas de Halson para la esterilización de las superficies vegetales y una combinación de Rifamicione y Benlate fue el más efectivo suplemento del medio. Esta técnica resultó útil para la colecta de germoplasma vegetal de las dos especies. En pruebas en el campo, 75% de los cultivos de* D. eriantha *ssp.* pentzii *y* C. dactylon *fueron asépticos y crecieron durante 4 semanas en cultivo. Los cultivos fueron transferidos al suelo bajo condiciones naturales y aproximadamente 70% de las 2 especies fueron recuperadas como plantas en macetas. En una prueba en el campo con* C. dactylon, *92% de los cultivos con raices y 80% de los cultivos sin raices en el momento del trasplante al suelo fueron éxitosamente establecidos como plantas en macetas.*

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Introduction

The grasses *Cynodon dactylon* and *Digitaria eriantha* ssp. *pentzii* (previously *D. decumbens*) are important forage species in tropical and subtropical pastures (Bogdan 1977). Their exploitation for livestock feed and soil protection will be enhanced if their genetic diversity is maintained by collection and conservation. A major constraint with these vegetatively propagated species, is poor seed production and germination. They are conventionally collected in the form of vegetative cuttings which deteriorate quickly and are subject to strict quarantine regulations in most countries.

In vitro culture techniques can be used to obviate these problems (IBPGR 1984). Vegetative material can be collected and transported as living cultures which reduce bulk, losses and transportation costs. Furthermore, cultures are subject to less stringent quarantine regulations. The wider applications of these techniques include the collection of immature embryos, where mature seeds are not present, and the collection of gametes.

Whilst *in vitro* collection techniques can be used routinely in fully equipped laboratories, suitable techniques for germplasm collection in the field using minimum facilities have only been reported for cacao (Yidana *et al.* 1987) and wild species of *Gossypium* (Altman *et al.* 1987). Minimum facility methods are invaluable for collection in developing countries where *in vitro* culture facilities are seldom available. Other suitable techniques have been reported for the collection of palm (Sossou *et al.* 1987) and coconut (Assy Bah *et al.* 1987) using makeshift laboratories in the field. No *in vitro* culture technique has been reported for the collection of forage grasses.

The objective of this study was to develop a simple method (minimum facility method (MFM)) for collecting *D. eriantha* ssp. *pentzii* and *C. dactylon* vegetative material using *in vitro* culture techniques outside the laboratory. This would be achieved by;

i) identifying a suitable agent for surface-sterilizing plant material in the field,

ii) identifying suitable antimicrobial agents to supplement the culture medium so as to reduce microbial contamination without adversely affecting the growth of cultures,

iii) inoculating surface-sterilized plant material into the medium under ambient conditions in the field,

iv) keeping the cultures growing for 4 weeks under uncontrolled conditions, and

v) transferring the cultures to the soil and growing the plants under natural conditions.

Materials and methods

Preparation of plant material

D. eriantha ssp. *pentzii* (ILCA No. 9729) growing at the Zwai seed multiplication site of ILCA was used for the development of the methods and the first field trial. *C. dactylon* growing wild at 5 randomly selected sites in the Shewa region of Ethiopia was used for the final field trial which was a simulated collection mission. All the work with plant material was carried out under ambient conditions outside the laboratory;

i) leaves were removed to reveal the axillary buds on stem cuttings,

ii) the stem cuttings were washed in boiled water and sterilized by immersing in a solution of surface-sterilization agent and shaking frequently for 30 minutes,

iii) the stem cuttings were excised into nodal cuttings of about 0.8 cm internode on either side of the node,

iv) the nodal cuttings were inoculated into 14 ml screw-capped glass vials containing 4 ml of semi-solid medium, and

v) the vials were lightly closed to allow gaseous exchange.

Preparation of culture medium

Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 1.07×10^{-7}M NAA, 2.22×10^{-7}M BA, 15 g/l sucrose and 10 g/l agar as used for the growth of *in vitro* cultures of *D. decumbens* (Ruredzo and Hanson 1990) was used as the culture medium. The medium was further supplemented with locally available antimicrobial agents (listed below) and the pH was adjusted to 5.6. The medium was then dispensed into screw-capped vials and autoclaved at 121 °C for 15 minutes.

Choice of surface sterilization agent

A suitable agent for surface-sterilizing plant material was selected during preliminary investigations from water tablets and the fungicides Banrot, Benlate, Canesten and Mycota (Appendix 1). Twenty pieces of *D. eriantha* ssp. *pentzii* leaf blades (1-cm long) were immersed in each of 0.1, 0.5, 1.0 and 5 g/l solution of these for 30 minutes. Five pieces were placed in each petri dish containing nutrient agar or malt extract and incubated for 5 days at 23–29 °C. The plant materials were scored for the presence of bacterial and fungal contamination. The treatment with the minimum overall contamination at the lowest concentration was chosen as the sterilization agent for the rest of the work.

The chosen sterilization agent was further tested similarly at 0.01, 0.1, 1.0, 1.5 and 2 g/l (active ingredient) and the weakest effective concentration chosen for the rest of the work. The sterilization agent was subsequently prepared by dissolving in boiled water. In order to reduce microbial contamination, boiled water was prepared by pouring boiling, locally obtained water into a screw-capped plastic can, closing the can immediately, and allowing the water to cool to ambient temperature.

Choice of antimicrobial medium supplements

Media containing seven different combinations of antibiotics (0.1 g/l) together with Benlate

(1.0 g/l) and one treatment without supplements were prepared and poured into 20 vials each. Nodal cuttings were prepared from *D. eriantha* ssp. *pentzii* shoots as described above, surface-sterilized and inoculated singly into the vials. The vials were kept under ambient conditions on a laboratory bench and scored after 4 weeks for the presence or absence of bacterial and fungal contamination, growth of cultures and necrosis (death for no apparent reason) by observing the individual cultures. The scores were compared to establish the best combination of antimicrobial agents which gave the highest level of asepsis and the lowest level of necrosis.

The most suitable concentration of the chosen antibiotic was established by culturing explants as above on media supplemented with 5 different concentrations (0.02, 0.04, 0.06, 0.08 and 1.0 g/l) whilst the concentration of Benlate was fixed at 1.0 g/l.

Field trials of the minimum facility method (MFM)

Two field trials were conducted independently to investigate the effectiveness of the chosen techniques using 200 cultures of *D. eriantha* ssp. *pentzii* plant material from the Zwai site and 40 cultures per site of *C. dactylon* from 5 randomly chosen sites in the Shewa region of Ethiopia. The overall recovery of potted plants and cultures, levels of bacterial and fungal contamination, levels of necrosis and the extent of spontaneous rooting of cultures were recorded to give a picture of the performance of the techniques. The performance of *in vitro* cultures and the resulting potted plants of wild *C. dactylon* from different sites was compared to assess the effectiveness of the technique on a simulated collection mission.

The best surface-sterilization agent and combination of antimicrobial supplements to the medium were used for the field trials of the MFM. Nodal cuttings of *D. eriantha* spp. *pentzii* were inoculated in the ambient conditions of a hotel veranda whilst those of *C. dactylon* were inoculated on site, on the open back of a pickup truck. The cultures were left on the laboratory bench under ambient conditions and individually scored by direct observation for viability, asepsis, bacterial and fungal contamination, necrosis and root formation after 4 weeks. The number of leaves, number of shoots, number of roots and the length of individual cultures were also recorded.

Living cultures were potted in locally purchased and untreated forest soil using a minimum facility method for transfer of *in vitro* cultures to soil (Ruredzo and Handson 1990): washing agar from the roots and planting the plantlets in forest soil in plastic pots which were then covered with plastic bags. To avoid dessication, humidity was kept high by maintaining some water in the plastic bags. After 2 weeks, the plastic bags were opened and the plants were regularly sprayed with water. The potted plants were removed from the plastic bags after 3 weeks when the plants were fully established. After a total of 4 weeks in the soil, the plants were scored for viability, number of leaves, number of shoots and the length of the longest shoot. Since individual cultures were followed throughout this work, it was possible to calculate the recovery of potted plants from cultures which were contaminated. It was also possible to calculate the recovery of potted plants from cultures which did not have roots and to investigate the effect of spontaneous rooting of *in vitro* cultures on the recovery of potted plants in natural conditions.

Scoring and analysis of results

The unit of observation in this work was the individual culture and the resulting potted plant. Plant vigor parameters (number of leaves, shoots, roots and plant height) were compared for living cultures and living potted plants only. Living potted plants from the field collection of *C. dactylon* were used to investigate the effect of roots on the establishment of plants from cultures.

SAS software (SAS 1985) was used for the analysis of the results. Where applicable, classificatory data (growth, asepsis, contamination, necrosis and rooting) were compared between treatments, collections and sites using Fisher's Exact Test (2-Tail). The General Linear Model was used for analysis of variance to compare quantitative data (number of leaves, number of shoots, number of roots and plant height).

Results

Surface-sterilization agent

Halazon water tablets at 1.0 g/l or above were the best surface-sterilizing agent. The other substances which were used had a fungicidal

Table 1. The effect of different combinations of antimicrobial agents on the performance of *in vitro* cultures of *Digitaria eriantha* ssp. *pentzii* after 4 weeks in culture

Supplements	*In vitro* cultures			
	Aseptic	Contaminated with bacteria	Contaminated with fungi	Necrotic
		(% collection[1])		
No supplements	15 b[2]	70 a	55 a	0 b
Rif + Rim	40 ab	25 b	45 ab	10 ab
Rif + Cot	45 ab	20 b	40 ab	20 ab
Rif + Ben	60 ab	30 b	20 b	10 ab
Rif + Cot + Ben	75 a	10 b	20 b	30 ab
Rif	35 b	20 b	60 a	5 b
Rif + Rim + Ben	75 a	10 b	15 b	35 a

[1] Some cultures were contaminated with both fungi and bacteria, hence the non-additivity of the percentages.
[2] Percentages followed by the same letter in a column are not significantly different ($P>0.05$), n = 20.

effect but succumbed to bacterial contamination giving erratic results. The concentration of water tablets which was adopted for the rest of the work was 1.0 g/l (active ingredient).

Antimicrobial medium supplements

A combination of Rifamycine and Benlate was the most effective antimicrobial supplement to the medium for *in vitro* collection of *D. eriantha* ssp. *pentzii*. Whilst this combination was poorer than 2 other combinations in terms of the recovery of aseptic cultures, it had significantly ($P<0.05$) fewer necrotic cultures (Table 1). Furthermore, this treatment ranked highest in the number of living cultures, growing cultures and mean number of leaves per culture, although differences between treatments were not significant ($P>0.05$).

The most suitable concentration of Rifamycine was 0.06 g/l (Figure 1) and so Benlate (1.0 g/l) and Rifamycine (0.6 g/l) were adopted for supplementing the medium.

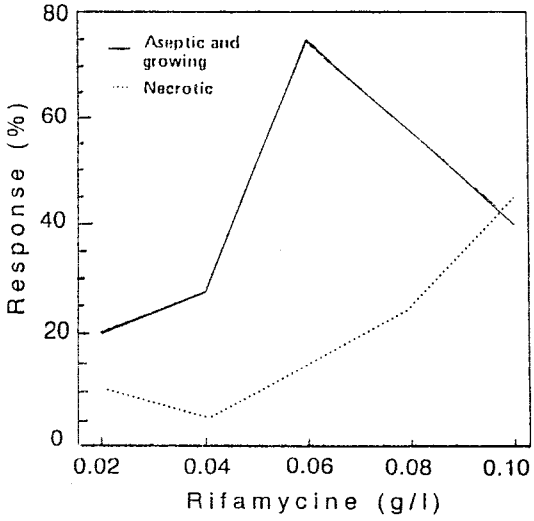

Figure 1. Performance of *D. eriantha* ssp. *pentzii* in medium supplemented with different concentrations of Rifamycine.

Field trials

Table 2 shows the performance of the 200 cultures each of *D. eriantha* ssp. *pentzii* and *C. dactylon* collected in the field. Although 78% of *D. eriantha* ssp. *pentzii* and 94% of *C. dactylon* were growing in culture when transferred to soil, only 74% and 72% of the 2 species, respectively, were successfully recovered as potted plants (Table 2). That is, 24% of the growing cultures of *C. dactylon* failed to continue growing when potted in soil. Some cultures which were contaminated (3% of the collection of *D. eriantha* ssp. *pentzii* and 9% of *C. dactylon* cultures), were successfully recovered as potted plants (Table 2). Eighty per cent of *C. dactylon* cultures which were planted without roots were able to root and grow in soil under natural conditions.

The numbers of growing cultures, necrotic cultures and cultures which were contaminated with both bacteria and fungi in the field collection of *C. dactylon* were not significantly different ($P>0.05$) between the collection sites (Table 3). Even so, the differences in overall plant recovery from the 5 collection sites were significant ($P<0.05$) and this appeared to be due to contamination by fungi and bacteria.

Table 2. Performance of *in vitro* cultures and resulting potted plants from *in vitro* field collection of *Digitaria eriantha* ssp. *pentzii* and *C. dactylon* after 4 weeks in culture and a further 4 weeks in soil (potted plants)

Response[1]	Cultures		Potted plants	
	D. pentzii	*C. dactylon*	*D. pentzii*	*C. dactylon*
		(% collection)		
Growing	78 b[2]	94 a	74 a	72 a
Aseptic	85 a	78 a	72 a	63 a
AG	74 a	76 a	72 a	63 a
Rooting	75 a	41 b	74 a	37 b
CB	8 a	10 a	2 b	7 a
CF	5 a	11 a	0 a	1 a
CFB	3 a	2 a	0 a	1 a
TC	16 a	22 a	2 b	9 a
Necrotic	7 a	3 a	0 b	10 a

[1] AG = Aseptic and growing, CB = Contaminated with bacteria, CF = Contaminated with fungi, CFB = Contaminated with both bacteria and fungi, TC = Total contamination.
[2] Percentages of cultures or potted plants followed by the same letter in a row are not significantly different ($P > 0.05$), n = 200.

Table 3. Performance of *C. dactylon* in culture and of resulting potted plants collected from 5 different sites using the *in vitro* minimum facility method

Response[1]	Site				
	1	2	3	4	5
			(% collection)		
Growing (cultures)	93 a[2]	100 a	90 a	88 a	100 a
Aseptic cultures	83 ab	78 ab	73 ab	65 b	92 a
AG	80 ab	78 ab	68 b	63 b	93 a
CF	13 ab	0 b	15 ab	25 a	0 b
CB	5 b	23 a	8 ab	5 b	8 ab
CFB	0 a	0 a	5 a	5 a	0 a
TC	18 ab	23 ab	28 a	35 a	8 b
Necrotic cultures	3 a	3 a	5 a	5 a	0 a
CCG	3 b	23 a	5 b	8 ab	5 b
Rooting cultures	20 b	75 a	20 b	25 b	63 a
Growing (pots)	75 a	90 a	50 b	55 ab	88 a

[1] AG = Aseptic and growing, CF = Contaminated with fungi, CB = Contaminated with bacteria, CFB = Contaminated with both bacteria and fungi, TC = Total contamination, CCG = Contaminated cultures which grew to plants.
[2] Percentages followed by the same letter in a row are not significantly different ($P > 0.05$), n = 40.

Living cultures from the field collection of *C. dactylon* from different sites did not differ significantly ($P > 0.05$) in the mean number of leaves per culture and mean shoot length but the mean number of shoots was significantly higher ($P < 0.05$) on shoots from site 5. Nevertheless, this difference was not reflected in potted plants which only differed in the mean length of the longest shoots which were significantly ($P < 0.05$) longer for plants from site 2 than for the other 4 sites (Table 4).

Cultures from the field collection of *C. dactylon* which had roots at the time of planting gave rise to significantly ($P < 0.05$) more viable potted plants than those which were planted without roots (92% and 80%, respectively; $P < 0.05$). Furthermore, plants which were planted with roots had significantly higher ($P < 0.05$) mean numbers of leaves, shoots and mean length of the longest shoot after 4 weeks in the soil (Table 5).

Discussion

The method developed here uses the minimum amount of materials and equipment for the collection of *D. eriantha* ssp. *pentzii* and *C. dactylon* in the field using *in vitro* culture techniques. The antimicrobial chemicals were bought in local

Table 4. Mean of growth parameters of *in vitro* cultures and resulting potted plants from the field collection of *C. dactylon*

Site	*In vitro* cultures		Potted plants
	No. of shoots	No. of roots	Shoot length
			(mm)
1	1.0 b[1]	0.3 c	54.6 b
2	1.2 b	2.6 a	105.8 a
3	1.2 b	0.5 c	63.2 b
4	1.0 b	1.0 cb	63.8 b
5	1.7 a	1.7 b	78.7 b

[1] Means followed by the same letter in a column are not significantly different ($P > 0.05$), n = 40.

Table 5. Means of growth parameters of potted plants derived from rooting and non-rooting *in vitro* cultures from the field collection of *C. dactylon*.

Plants from cultures	Growth parameters		
	No. of leaves	No. of shoots	Shoot length
With roots	7.5 a[1]	1.4 a	96.0 a
Without roots	5.8 b	1.1 b	55.7 b

[1] Means followed by different letters in a column are significantly different ($P < 0.05$), n = 72 with roots, 71 without roots.

pharmacies in Addis Ababa, Ethiopia. One hundred vials fitted into compartments of a 30 x 30 x 7-cm box. The basal medium, growth regulators and vials were imported and heat-sterilized in an autoclave. Even so, costs can be further reduced by using locally available glass jars instead of vials and a household pressure cooker instead of an autoclave. The method is therefore very suitable for use in the field by collectors with space or financial constraints. However, it needs to be validated with other grass species and the basal medium needs further experimentation to reduce costs.

The successful re-establishment of cultures to natural conditions in the soil out of the laboratory is the most important success indicator of a minimum facility method for collecting vegetative germplasm using *in vitro* culture techniques. Using this method, the overall recovery of plants in soil included cultures which were successfully transferred to soil despite contamination. These should only be released from the culture vessels where quarantine is not important or after using laboratory techniques to ensure asepsis. It is also shown in this study that cultures which had roots at the time of planting gave rise to significantly ($P<0.05$) more potted plants, therefore, plant recovery can be improved by either adding growth substances which promote rooting to the original medium or by applying such agents during transfer to soil.

Most of the living cultures after a collection mission can be recovered, especially where laboratory facilities are available. In this study, a much higher percentage of the collection was growing *in vitro* compared to that of recovered potted plants. Since laboratory techniques were not used to maximize plant recovery, it is very likely that plant recovery can be increased if the cultures are subjected to laboratory techniques after the collection mission is completed.

The overall acceptability of a medium by a particular species can be assessed by the amount of necrosis. The medium in this work was very acceptable to the species studied since the level of necrosis was low (7 and 3% of *D. eriantha* ssp. *pentzii* and *C. dactylon*, respectively) after 4 weeks in culture. Furthermore, in the field collection of *C. dactylon*, the differences in percentage necrosis (2–0%) between collection sites were not significant ($P>0.05$) indicating that the loss of particular genotypes through necrosis was unlikely.

Contamination in this work (15.5% and 22% of *D. eriantha* ssp. *pentzii* and *C. dactylon*, respectively) compared well with the 17% reported by Yidana *et al.* (1987). It is clear from the differences between sites in this work

(7.5–35% contamination) that the contamination levels were site-related. As demonstrated, these differences were due to high fungal contamination on two sites and high bacterial contamination on one site. Since most of the germplasm losses during the field trials were due to contamination, further investigations are necessary to ensure that there is no selection against susceptible genotypes. Losses could also be reduced, where possible, by planning collections using this method for the time when the microbial spore count is low.

In aseptic *in vitro* culture without the use of antimicrobial agents, contamination generally appears within the first few days of culture. In these cultures contamination appeared even after 2 weeks indicating that the antimicrobial agents might only inhibit or retard the growth of some micro-organisms but not eliminate them. It is recommended that collections should be subcultured on media without antimicrobial agents before they are assumed to be free from non-systemic micro-organisms. Where quarantine is important, heat therapy and meristem culture should be carried out and the cultures disease-indexed to ensure that they are free from specific disease-causing agents before they are released. The use of this method is therefore subject to quarantine regulations which should be observed especially for international germplasm exchange.

Conclusion

The forage species *D. eriantha* ssp. *pentzii* and *C. dactylon* can be successfully collected in the field using *in vitro* culture techniques and kept growing *in vitro* under ambient conditions for at least 4 weeks by surface-sterilizing the explants and inoculating in growth medium supplemented with antimicrobial agents. The resulting cultures can be successfully re-established in the soil under natural conditions outside a laboratory. Since the method was developed using one species and successfully applied to another, it is very likely, but still needs to be verified, that it is applicable to other grass species. Such methods are invaluable to germplasm collection in the field away from laboratory facilities. However, despite the flexible quarantine restrictions applied to *in vitro* cultures, this method should only be used for international germplasm exchange where suitable facilities are available at the destination for isolation, further checks and disease indexing.

Acknowledgements

The author was financed by IBPGR during the course of this work. Dr Jean Hanson, the head of the Forage Genetic Resources Section (ILCA), is gratefully acknowledged. Ato Mekonen Girma, the research assistant on this project, and ILCA staff who assisted with the preparation of the manuscript are especially thanked.

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Appendix 1. List of antimicrobial agents used

Name used	Common name	Active ingredients
Banrot	Banrot	5-ethoxy-3-trichloromethyl-1,-2,4-thiadiazole (15%) dimethyl 4,4-0-phenyl-enebis 3-thioallophanate (25%)
Ben	Benlate	Benomyl [methyl 1-butyl-carb-2-benzimidazole carbamate] (50% w/w)
Canesten	Canesten (broad spectrum antimycotic with fungicidal action)	2 ml solution contains 0.2 g of bis-phenyl (2-chloro phenyl)-1-imidozolyl methane
Cot	Co-trimoxazole	Trimethoprim 80 mg sulphamethoxazole 400 mg
Mycota	Mycota power for treatment and prevention of athlete's foot	Undecenoic acid B.P. 2% w/w Zinc undecenoate B.P. 20% w/w
Rif	Rifamycine Chibret (collyre eye-drops)	Rifamycine SU monosodium salt
Rim	Rimactan 300	Rifamycine 300 mg/capsule
Water tablets	Halazon tablets for water treatment	p-carboxybene sulphon-dichloro-amide 4 mg/tablet

(Received for publication, October 13, 1990; accepted December 28, 1990)