

Embryogenic Cell Suspensions From Different Explants and Cultivars of *Eragrostis curvula* (Schrad.) Nees

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ABSTRACT: The aim of this work was the establishment of embryogenic calli and cell suspensions from different explants and cultivars of weeping lovegrass, *Eragrostis curvula* (Schrad.) Nees, to be used as targets for biolistic transformation.

Calli were initiated from immature inflorescences, seeds, embryos, leaf bases and root tips. Modified MS medium (Murashige and Skoog, 1962) was used for calli induction and proliferation. Cell suspensions were established and maintained in AAF medium (Wang *et al.*, 1993).

Morphogenic calli, embryogenic cell suspensions of moderate growth rate - consisting mainly of compact proembryogenic cell clusters- and green plants were obtained from all the explants and cultivars assayed, except root tips.

Both, explant and genotype were very important factors to be considered in order to obtain a morphogenic response and to establish cell suspensions from this grass. The statistical analysis detected interaction between both factors, explants and genotypes. Immature inflorescences were the best source of explant and Kromdraai was the cultivar that showed the best morphogenic response (expressed as the percentage of calli/explant and the percentage of calli with green spots -every green spot developed into green plants-) with inflorescences, embryos and leaf bases. For Morpa and Don Pablo embryos as explants were less responsive than seeds and leaf bases. There were no differences in leaf bases for all the three cultivars analysed.

Abbreviations: AA medium- medium described by Müller and Grafe (1978); AAF medium-AA medium modified by Wang *et al.* (1993); cv-cultivar; 2,4-D- 2,4-dichlorophenoxyacetic acid; MS- Murashige and Skoog medium.

Introduction

For plant genetic engineering to be successful, an effective method for growing transformed cells into fertile plants is an absolute requirement (Jähne *et al.*, 1995). In many major crop plants, however, this step still pre-

sents difficulties, particularly, the absence of a working regeneration protocol. Even if a regeneration protocol has been established for one plant variety, it can rarely be applied unchanged to another. It is not only the genotype which plays a decisive role, but also the plant tissue culture system itself. For a long time, cereals seemed to be recalcitrant in tissue culture. However, considerable progress has recently been made in establishing reliable and efficient *in vitro* culture systems (Vasil, 1988; Wang *et al.*, 1995). The success in obtaining regenerating cultures of several plant species which were once regarded recalcitrant, such as cereals, grain le-

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gumes and forest tree species, has been possible largely due to a shift in emphasis from media manipulation to explant selection (Bhojwani and Razdan, 1996). Now, embryogenic cell suspensions can be used as targets for biolistic transformation (Wang *et al.*, 1994). They are also a unique source of totipotent protoplasts for graminaceous species (Stadelmann *et al.*, 1998).

Weeping lovegrass *Eragrostis curvula* (Schrad.) Nees is an important forage grass for semiarid regions. It has important traits such as drought resistance and a perennial habit (Covas, 1991). One objective for improvement in this grass is to increase digestibility. However, the reproductive mode -apomixis- restricts the use of conventional breeding methods. For this reason, plant transformation is an attractive alternative. In a previous paper we described a method to regenerate fertile plants from immature inflorescences in five cultivars of *E. curvula* (Echenique *et al.*, 1996). Inflorescences are excellent explants but non available all through the year. The aim of this work was to establish embryogenic cell suspensions from different explants and cultivars of *Eragrostis curvula*.

Materials and Methods

Plant material:

Three cultivars of *Eragrostis curvula* (Schrad.) Nees: two full apomicts (Morpa and Don Pablo) and a facultative one (Kromdraai) were compared, and seeds, embryos, leaf bases, immature inflorescences and root tips were used as explants.

Culture media:

For calli induction, MS medium (Murashige and Skoog, 1962) supplemented with 2 mg l⁻¹ 2,4-D, 0.01 mg l⁻¹ BAP, 500 mg l⁻¹ casein hydrolysate, 3% sucrose (w/v) with and without 0.5% agar (w/v) (purified, Sigma A-7921) was used. For calli proliferation, friable, yellowish, embryogenic calli were subcultured on MS medium supplemented with 1.0 mg l⁻¹ 2,4-D, 400 mg l⁻¹ casein hydrolysate, MS vitamins and 0.5% agar (w/v) (Wang *et al.*, 1993). In both media, pH was adjusted to 5.8 before autoclaving for 20 min at 1 atmosphere.

Inflorescences culture:

Inflorescences just emerging from the flag leaf of plants growing in the greenhouse were surface steril-

ized in 70% ethanol for 1 min, followed by commercial bleach (0.8% of active chlorine) with 2 drops of Tween 20 for 15 min, and washed three times with distilled water. Inflorescence segments 1cm long were cultivated in test tubes (25 x 150 mm) (3 explants/tube) and in Petri dishes (100 x 15 mm) (5 explants/dish) containing 15 and 20 ml of induction medium, respectively, for four weeks in the dark at 25°C. Then, calli were transferred to proliferation medium and cultured under dim light (7-11 μmol m⁻² s⁻¹) for 1 month and finally put under normal light conditions (66 μmol m⁻² s⁻¹).

Seeds and embryos culture:

Mature seeds were surface sterilized in 70% (v/v) ethanol for 1 min, followed by commercial bleach (8% of active chlorine) with 2 drops of Tween 20 for 20 min. They were then rinsed 3 times with sterile distilled water and 24 h soaking in sterile water. Then, after a re-sterilization in commercial bleach (12% v/v), seeds were soaked in disposable Petri dishes (20 seeds/dish) and in test tubes (5 seeds/tube). In a similar way, seeds were excised and the embryos were soaked and cultured as the seeds. Minimum sample size was 400 seeds or embryos/cultivar/treatment.

Leaf bases and root tips culture:

Seeds were surface sterilized as above and plated in Petri dishes with wet paper and cotton to promote germination. After 10 days, root tips and leaf bases were excised at 2 mm from the tip and at 2 mm from the seed, respectively.

Both explants were plated in Petri dishes and test tubes and cultured as the other explants assayed. Minimum sample size was 400 explants/cultivar/treatment.

Cell suspensions:

Embryogenic calli growing in proliferation medium were transferred to AAF liquid medium, AA medium (Müller and Grafe, 1978) containing 2.0 mg l⁻¹ 2,4-D, 2% sucrose, 3% sorbitol, 10 ml l⁻¹ B5 vitamins (Wang *et al.*, 1993), and to a medium with the same composition but replacing AA by MS macronutrient salts.

Cell suspensions were cultured in 125 ml Erlenmeyer flasks with 30 ml of liquid medium under continuous shaking on an orbital shaker at 80 rpm. For the first week they were kept in the dark at 25°C, before moving to dim light (7-11 μmol m⁻² s⁻¹). Culture medium was replaced every 2 weeks.

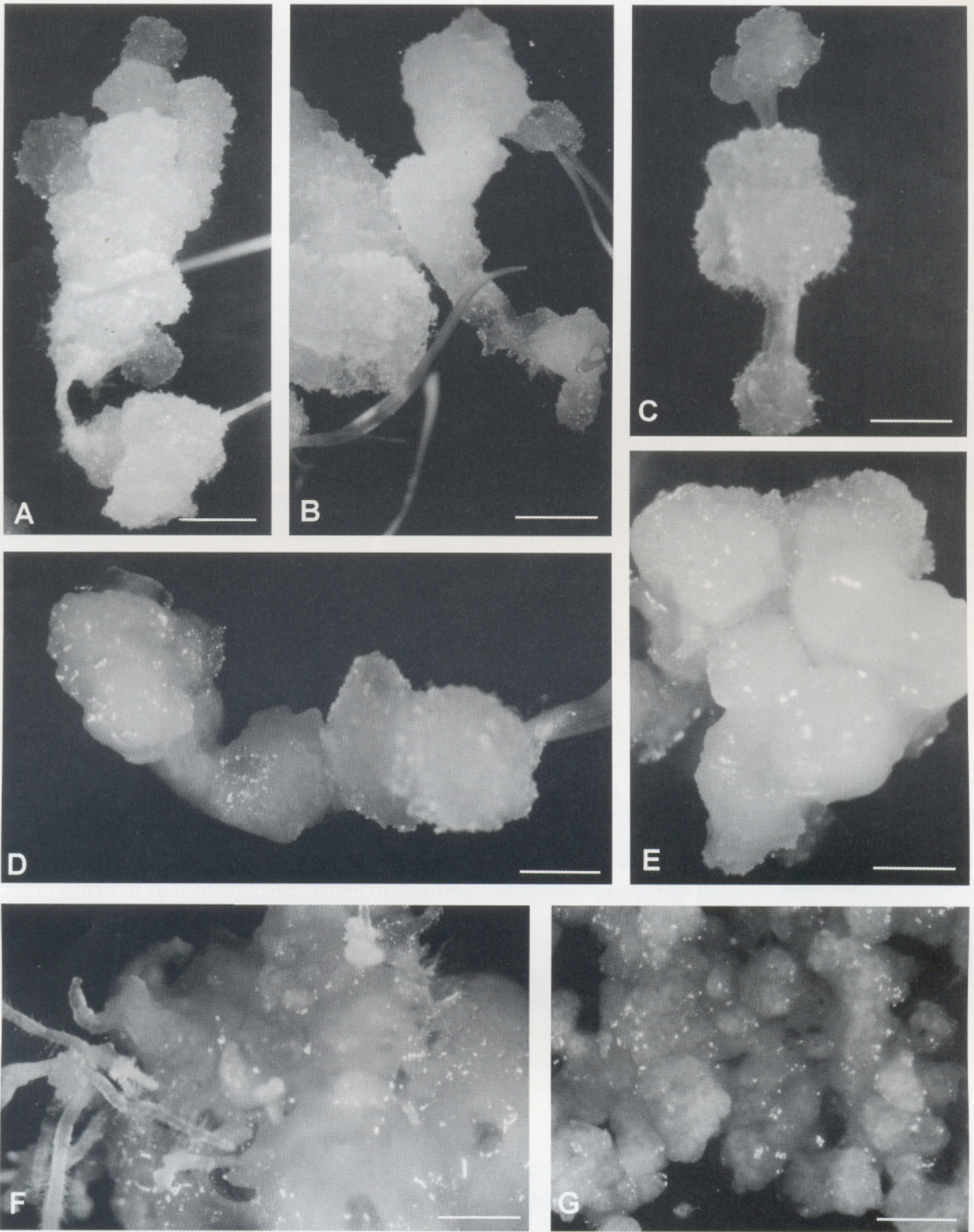


FIGURE 1. Callus induction in *Eragrostis curvula* by *in vitro* culture of different explants. Leaf base callus from cv. Tanganika (A). Germinated seed with white soft callus surrounding the shoot from cv. Tanganika (B). Leaf base callus (C). Seed derived Morpa callus (D and E). A plated cell suspension from Morpa cultivar. (G). Bars equals: 0.3 cm in figures A, B and D, 0.42 cm in figures C, E and G and 0.75 cm in figure F.

When small clusters were transferred to a solid MS medium with 3% sucrose, without growth regulators, plant regeneration was initiated.

Plant regeneration capacity was determined as the percentage of calli/explant and the percentage of calli with green spots. Every green spot developed into green plants.

Statistical Analysis:

A completely randomized design was used. Each Petri dish or test tube was considered as an experimental unit. ANOVA and Tukey's tests were carried out with the SYSTAT 7.0 program.

Results and Discussion

The protocol for weeping lovegrass regeneration described in this paper was developed from a series of experiments where we investigated the potential of leaves, roots, inflorescences, seeds and embryos to regenerate plants. All the explants sources produced some calli after 4-8 weeks in culture. Root tips started to form small non-embryogenic calli that grew very slowly. Seeds gave rise to callus masses in the areas surrounding the embryo and also typically germinated, and cell proliferation on the shoot formed a white wet callus (Fig. 1B and D). No differences were observed between embryos and seeds in callus morphology. These calli were mainly soft, aqueous and with a higher ratio of non-embryogenic tissue. Twenty days after starting the cultures, the embryos developed creamy yellow soft calli with mucilaginous consistency (Fig. 1E and F). Embryogenic tissue appeared between soft and amorphous calli and developed into somatic embryos. Green points appeared 5 days after transfer to light.

Leaf base calli were soft, aqueous and mainly non-embryogenic (Fig. 1A and C), but it was possible to regenerate plants from small embryogenic spots immersed into the non-embryogenic callous masses. Mekbib *et al.* (1997) were able to regenerate plants from *Eragrostis tef* leaf base using dicamba as growth regulator. Bekele *et al.* (1995) also working with *Eragrostis tef* found that, on the whole, the number of regenerants from leaf callus was higher than that from root callus at all the tested hormone concentrations.

Statistical analysis detected interaction between explants and genotypes ($p < 0.01$). With leaf bases as explants no differences were observed between genotypes, having similar response than embryos in Kromdraai and seeds in Morpa and Don Pablo (Tables 1 and 2). But

leaf base is not an explant suitable to start cell suspensions or on which to base an efficient regeneration system for *Eragrostis curvula*.

Cv. Kromdraai had the best response in embryogenic callus formation and plant regeneration with all the explants assayed, except with seeds, that were less responsive (Fig. 2, Table 1). Calli in this cultivar were friable and embryogenic (Fig. 2B) and the 100% of them developed green spots that gave rise to healthy plants (Fig. 2 and 3). With the same explant source, Morpa and Don Pablo calli appeared later in culture, between 15 to 30 days. For these two cultivars, embryos were the less responsive explants (Tables 1 and 2).

Inflorescences were analysed separately because the experimental units were different. This was because the culture vessel was also important in the *in vitro* response. Inflorescences produced friable embryogenic calli when culture was initiated in test tubes (Fig. 2A). In 30 days it was possible to observe small callus masses that grew fast to produce friable embryogenic calli. When these calli were transferred to light conditions it was possible to observe green spots distributed in all over the mass (Fig. 2B). If inflorescences are cultured in Petri dishes the response is very different, even for the same genotype. Sometimes there is no response or it takes more time to produce small calli, at least 2 months. Embryos and seeds respond in a different way. When these explants are cultivated in test tubes, the calli are mainly aqueous and non-embryogenic. A better response is observed in Petri dishes, with a higher proportion of embryogenic to non-embryogenic calli.

Our results indicate that immature inflorescences are the most responsive tissue to obtain calli, regenerate plants and also to initiate cell suspensions from this grass. Immature inflorescences have been recognized as an important source of totipotent cultures in many cereals and grasses (Wernicke and Brettell, 1980; Jackson *et al.*, 1986). Areas of white, compact callus, typical of graminaceous embryogenic callus, developed in the middle of aqueous non-embryogenic calli (Fig. 2A). Cell proliferation appeared to be from tissue within the individual florets and did not involve the stem (rachis and rachilla) or bract tissues (Fig. 2A, arrows). Only the calli from floret primordia showed morphogenic potential (Echenique *et al.*, 1996). Straub *et al.* (1992) found similar results in *Sporobolus virginicus*. They stated that the initial cell proliferation appears to be confined to the ovary and did not involve the stamens or the surrounding bracts.

Cell suspension cultures were initiated from compact calli derived from seeds, embryos and inflorescences. These cultures varied considerably in their

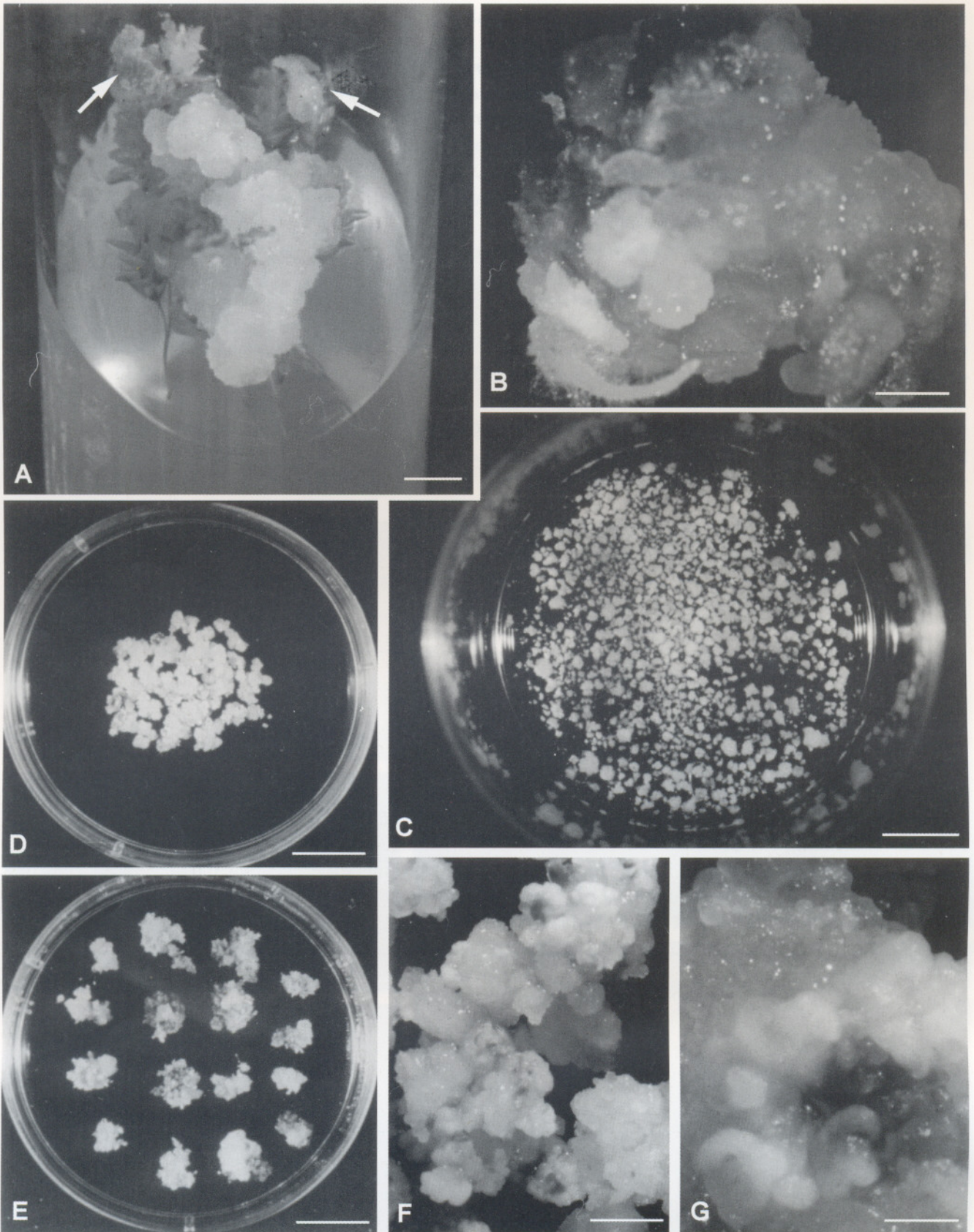


FIGURE 2. Calli obtained from immature inflorescences, cv. Kromdraai (A and B). Arrows indicate cell proliferation from florets. A cell suspension derived from inflorescence calli, cv. Kromdraai (C). Plated clumps from the cell suspension showed in C, different stages (D, E, F and G). Bars equals: 0.35 cm in figure A, 0.20 cm in figure B, 1.86 cm in figure C, 5.34 cm in figures D and E, 0.6 cm in figure F and 0.09 cm in figure G.

TABLE 1.

Effect of explant sources for callus production and plant regeneration in different cultivars of Eragrostis curvula (Schrad.) Nees.

Note: Results are expressed in % from 400 explants/treatment and are expressed in %. All of the green spots developed into healthy plants.

EXPLANT	cv. Kromdraai	cv. Morpa	cv. Don Pablo
Inflorescences			
Calli/ 100 explants	97	88.02	70.23
Calli with green spots/ 100 calli	100	95.23	76.75
Seeds			
Calli/ 100 explants	87.5	98.8	95.26
Calli with green spots/ 100 calli	15.62	52.17	18.33
Embryos			
Calli/ 100 explants	95	90	55
Calli with green spots/ 100 calli	50	45	34
Leaf bases			
Calli/ 100 explants	95	97.15	97.11
Calli with green spots/ 100 calli	50	45	60
Root tips			
Calli/ 100 explants	0	0	0.5
Calli with green spots/ 100 calli	0	0	0

TABLE 2.

Average of the number of explants that produced calli per experimental unit in the different cultivars.

Note: Means followed by different letters are significantly different at the 0.05 level of probability in seeds, embryos and in cv. Kromdraai and at the 0.01 level of probability in inflorescences, cv. Morpa and Don Pablo according to Tukey's test.

EXPLANT	cv. Kromdraai	cv. Morpa	cv. Don Pablo
Inflorescences	2.896 c	2.615 b	2.096 a
Seeds	17.5 a	19.8 b	19.1 b
Embryos	19.0 c	17.75 b	11 a
Leaf bases	19.0 a	19.45 a	19.45 a
CULTIVAR	Seeds	Embryos	Leaf bases
Kromdraai	17.5 a	19.0 b	19.0 b
Morpa	18.8 b	17.75 a	19.45 b
Don Pablo	19.10 b	11.00 a	19.45 b

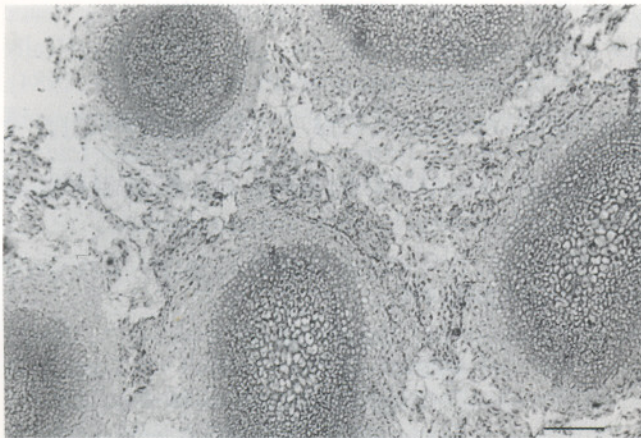


FIGURE 3. Histology of cell suspension clumps, cv. Kromdraai. Bar equals: 200 μ m.

growth response. Regenerable cell suspensions of moderate growth rate, consisting mainly of compact medium sized (2-3 mm) proembryogenic cell clusters (Fig. 2C) were established for cultivars Morpa, Don Pablo and Kromdraai cultivars using suspension culture medium AAF (Wang *et al.*, 1993). The histological study showed embryogenic masses surrounded by non embryogenic tissue (Fig. 3). When small clusters were transferred to a solid MS medium with 3% sucrose without hormones plant regeneration was initiated (Fig. 2D, E, F and G).

It was possible to observe differences between cell suspensions obtained from seed or embryo calli and from inflorescences, and also between cultivars. Cell suspensions from seed or embryo calli grew slower than those from inflorescences, showing some degree of oxidation in seed or embryo-derived ones (Fig. 1F and G).

Although seeds and embryos are less useful explants than inflorescences, it compensates by being available all through the year. Seeds are always avail-



FIGURE 4. Plantlets from a plated cell suspension, cv. Kromdaai (A, B and C). R plants flowering in the field (D and E). Bars equals: 0.75 cm in figures A and C, 1.71 cm in figure B.

able and render the whole procedure simpler and less time consuming.

Oxidation was observed when MS based medium was used for the establishment and maintenance of suspension cultures of the same three cultivars. When plated on regeneration medium, apart from the formation of somatic embryos in restricted areas, the developing of roots was observed (Fig. 1F). A similar effect was observed in *Eragrostis tef* embryogenic callus without an apparent relationship with the culture media (Kebebew *et al.*, 1998).

We also attempted to establish cell suspensions from seeds and embryos growing from the beginning in liquid MS medium, but the callus morphology is better if the cultures are initiated in solid medium.

Regenerated plants (Fig. 4 A, B and C) were planted in pots with soil and transferred to the greenhouse. Plants were robust, grew rapidly, flowered and set seed. Seeds from R₀ plants were sown in the field and also flowered and set seeds (Fig. 4 D and E).

Plant regeneration from a tissue culture system is

often the most critical step in a plant improvement program. Embryogenic cell lines maintain their competence for a long period of time, and give rise to genetically uniform and normal plant populations (Vasil *et al.*, 1984). The establishment of morphogenic cell suspensions should help in the further application of gene transfer technologies into this grass, trying to achieve stable genetic transformation by particle bombardment.

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