In Vitro Cell. Dev. Biol.—Plant 40:299–302, May–June 2004
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1054-5476/04 $18.00+0.00

DOI: 10.1079/IVP2003533

REGENERATION OF BLACK GRAMA (*BOUTELOUA ERIOPODA* TORR. TORR) PLANTS VIA SOMATIC EMBRYOGENESIS

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(Received 29 April 2003; accepted 23 December 2003; editor P. Ozias-Akins)

Summary

Black grama (*Bouteloua eriopoda*) is an important forage grass in southwestern USA rangelands. Plants were regenerated by somatic embryogenesis. Surface-disinfested seeds were germinated and the embryonic shoots were excised and cultured on Murashige and Skoog (MS) medium gelled with agar. Callus was induced from apical meristems. Calluses were cultured on MS solid medium with six concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) or Dicamba (6-dichloro-*o*-anisic acid) for 6 wk under light or dark conditions. Somatic embryo induction was greatest on 4.52 μM Dicamba, under light, after transferring to an auxin-free medium. Embryo development progressed from globular torpedo to mature embryos phenotypically identical to those naturally produced in seed. These germinated and grew into intact plants and were established in soil and grown to maturity. To our knowledge, this is the first report of somatic embryo induction and regeneration in black grama grass.

Key words: arid; drought; endophyte; range grass; symbiosis.

Introduction

Black grama, *Bouteloua eriopda* (Torr.) Torr., a C-4 warm-season perennial, is an important native forage grass in arid southwestern USA rangelands. Its drought resistance and dominance in the northern Chihuahuan Desert make this grass a practical ecological and physiological research plant (Mckell and Goodin, 1973).

Somatic embryos in cereals and grasses originate exclusively from meristematic cells (Wernicke and Brettel, 1980; Wernicke et al., 1982; Wernicke and Milkovits, 1984). Somatic embryos may arise from *in vitro* culture of mature seed (Sahasrabudhe et al., 1999; Selles et al., 1999), immature embryos or spikes (Vasil and Vasil, 1982; Li and Qu, 2002), meristems and apical meristems (Bhaskaran and Smith, 1990; Osuna-Avila et al., 1995). Exogenous auxins, such as Dicamba (6-dichloro-*o*-anisic acid), Picloram (4-amino-3,5,6-trichloropicolinic acid), 2,4-dichlorophenoxyacetic acid (2,4-D), or 2,4,5-tricholorophenoxyacetic acid (2,4,5-T) are needed for induction of somatic embryos in numerous plant species (Kearney et al., 1991). Whole plants can also be regenerated from somatic cells of some plant species without auxins (Vasil, 1987). Aguado-Santacruz et al. (2001b) induced somatic embryos from meristematic cells of shoot apices of blue grama (*Bouteloua gracilis*), a closely related species to *Bouteloua eriopoda*, by culturing surface-disinfested seedlings on MS medium (Murashige and Skoog, 1962) supplemented with 4.52 μM 2,4-D, 8.87 μM benzylaminopurine (BA), and 296.08 μM adenine. Osuna-Avila et al. (1995) induced somatic embryos from shoot meristems of *Setaria italica* on MS medium supplemented with 2,4-D or 2,4,5-T.

The objectives of this study were to test different growth regulator types and concentrations for somatic embryogenesis induction to regenerate plants from apical meristematic cells of germinating *B. eriopoda* seedlings.

Materials and Methods

Seeds of *Bouteloua eriopoda* were harvested from native populations on the USDA-ARS, Jornada Experimental Range, in the northern Chihuahuan Desert. Seeds were surface-disinfested by soaking in 75% ethanol for 8 min under 0.28 kg cm^{-1} vacuum in Chlorox (80%; 5.25% sodium hypochlorite, active ingredient) for 15 min and rinsed several times with sterile distilled water. Disinfested seeds were germinated on MS salts and vitamins (Murashige and Skoog, 1962) supplemented with 3% sucrose (w/v). The pH was adjusted to 5.8 and the medium was solidified with 1.5 g l^{-1} of phytagel (Sigma, St. Louis, MO). Media were autoclaved at 121°C for 20 min.

Callus and embryo induction. Disinfested seeds were germinated on MS medium. After 1 wk, embryonic shoots approximately 1 cm long were excised at the root junction and cultured on MS medium, prepared as above, with the following treatments for callus induction. The experimental design consisted of two auxins, 2,4-D or Dicamba, each at six concentrations of 4.52, 9.05, 13.57, 18.1, 22.62, and 27.14 μM. In two light treatments, apical meristems were cultured in continuous dark or under an 18 h photoperiod (151 μmol m^{-2} s^{-1}) at room temperature (25°C). After 4 d, calluses formed at the base of the excised shoots. Calluses were removed and subcultured on their respective initiation media, 10 per Petri plate (9 cm), with 10 replicate plates per treatment for 52 d. At this time, the number of shoots that produced proembryoids per total number of seeds was calculated. A one-way ANOVA was used to determine treatment responses of plant regeneration. Mean differences were determined by Tukey's mean separation.

Plant regeneration. From each treatment, 12 replications of 500-mg pieces (fresh weight) of embryogenic calluses (EC) were transferred to auxin-free MS medium and placed in a growth chamber at 25°C with a 16 h (151 μmol m^{-2} s^{-1}) photoperiod for 6 wk. The number of complete plants were recorded and differences in plant regeneration between treatments

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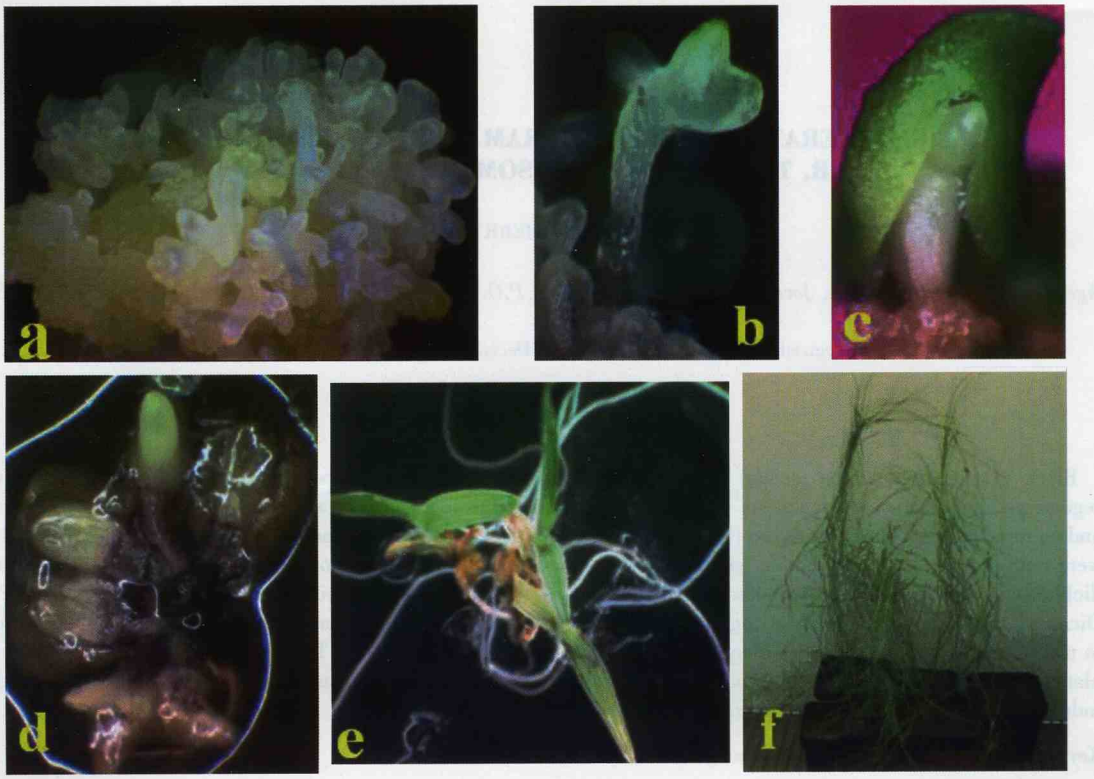

FIG. 1. Somatic embryogenesis in the culture of shoot apices of black grama. *a*, Embryogenic callus with proembryoids. *b*, Embryo. *c*, *d*, Secondary embryos. *e*, Plant elongation. *f*, Established plants.

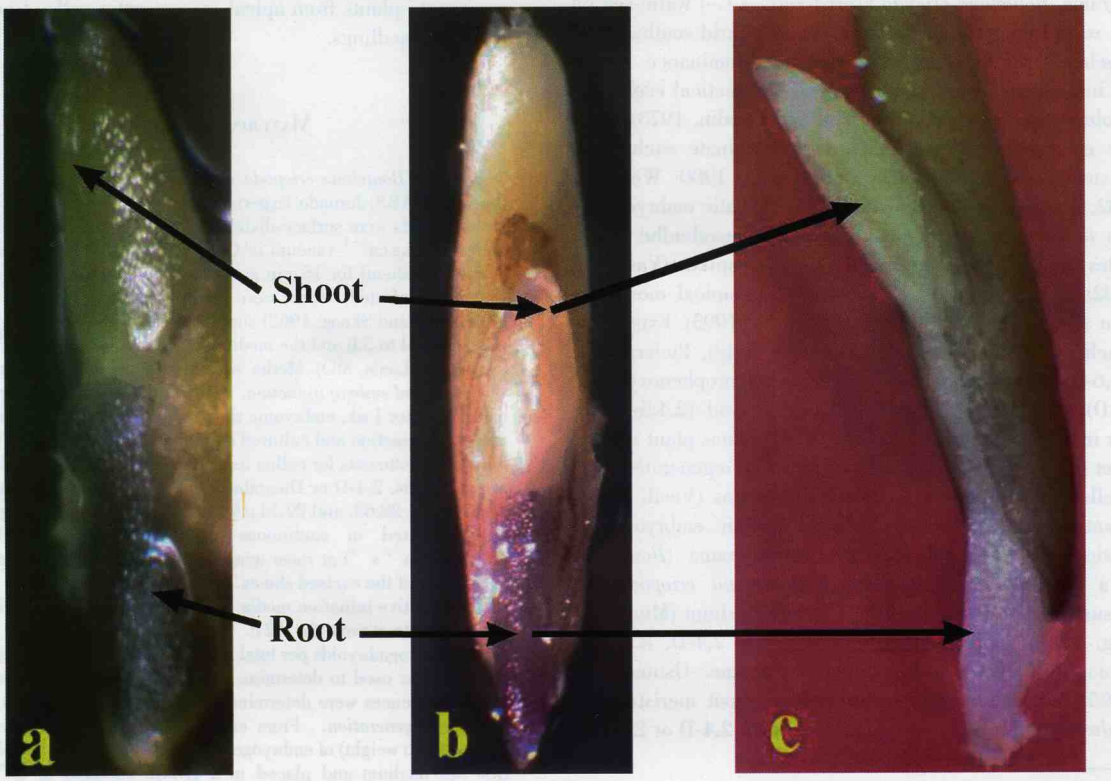

FIG. 2. Resemblance between black grama embryos. *a*, Somatic embryo. *b*, *c*, Sexual embryos from caryopses 24 and 50 h old, respectively.

were determined using a one-way ANOVA and significant differences between means were assessed by Tukey's mean separation.

Greenhouse establishment. Mature plants with three to four leaves and an adequate root system (primary and secondary roots) were transferred into pots (12.7 cm) filled with sandy soil from native black grama sites on the Jornada Experimental Range. Pots were placed in a shaded greenhouse and covered with plastic bags for 2 wk to prevent desiccation.

Results and Discussion

Callus proliferated within 3–4 d from all shoot explants in all auxin concentrations and exposure to both light and dark conditions. Similar callus formation occurred in *Setaria italica* (L.) tissue cultures (Osuna-Avila et al., 1995) and in *Bouteloua gracilis* (Aguado-Santa Cruz et al., 2001b). In preliminary studies, *B. eriopoda* embryonic shoots cultured on the medium developed by Aguado-Santa Cruz et al. (2001a, b) for *B. gracilis*, became necrotic and produced no calluses. Differences may be attributed to media since they used MS medium supplemented with 2,4-D (4.52 μM), BA (8.87 μM), and adenine (296.08 μM), while we used only auxin (2,4-D or Dicamba).

Two types of calluses were observed after 52 d of culture. A white, compact, nodular callus with embryonic structures (globular and torpedo stages) that developed on the callus periphery and was classified as embryogenic callus (EC) (Fig. 1*a*). Early-stage embryos continued to develop (Fig. 1*b*, *c*). A yellow, soft, wet callus lacking proembryoid structures was classified as nonembryogenic callus (NEC).

Somatic embryo production was most frequent on 4.52 μM Dicamba and progressively decreased on 9.05 and 13.57 μM. No somatic embryos developed in the dark at auxin concentrations above 18.1 μM (Table 1). A higher frequency of somatic embryo induction was obtained in this study (37.1% for 2,4-D and 58.2% for Dicamba) compared to less than 3% in *B. gracilis* explants reported by Aguado-SantaCruz et al. (2001a, b). Variable percentages for embryo induction were observed between seedlings, indicating a possibility for intraspecific genetic variability within cross-pollinated native populations, while differences between *B. eriopoda* and *B. gracilis* may be due to interspecific variability.

The lowest auxin concentrations yielded the highest frequency of embryos (Table 1), indicating that auxin levels below 4.52 μM may or may not result in increased embryogenesis. Embryogenic calluses initiated with 4.52 μM Dicamba and cultured under light were the best sources for plant regeneration, yielding around 20 plants per 500 mg (fresh weight) callus (Table 2). The stage of embryo development upon transferring to auxin-free medium could be critical for efficient plantlet development (Bhojwani and Razdan, 1983). Further experimentation will be necessary to synchronize developmental stages to maximize regenerated plant production for large-scale experiments.

When mixed EC and NEC were transferred to an auxin-free medium, the NEC gave rise to either shoots without attached roots or roots without attached shoots. These organs resulted from organogenesis and failed to produce complete plantlets. Organogenic calluses formed organs (shoot or roots) which may be adventive or *de novo* origin (Phillips et al., 1995). Complete plants were regenerated from somatic embryos that were phenotypically similar to zygotic embryos from seed (Fig. 2). Four days after transfer to the auxin-free medium, green sectors began to develop within the EC and were completely green after 15 d. Compact EC produced embryos in 20 d and secondary embryos were also frequently observed. Mature bipolar embryos removed from EC and transferred to auxin-free medium began to root after 3 d and developed a good root mass and three to four leaves, 1 cm long, after 13 d (Fig. 1*e*). Approximately 75% of the plantlets survived transplanting into pots containing sandy soil taken from native *B. eriopoda* sites on the Jornada Experimental Range and grew to

TABLE 1

EFFICIENCY OF SOMATIC EMBRYO INDUCTION IN 16 TREATMENTS IN *BOUTELOUA ERIOPODA* SHOOTS CULTURED UNDER LIGHT AND DARK CONDITIONS FOR 52 d

Incubation conditions	Auxin ($mg l^{-1}$) 2,4-D	Auxin ($mg l^{-1}$) Dicamba	Somatic embryo induction (%)
Light	4.52		37.1 b
	9.05		16.0 e
	13.57		10.0 g
	18.10		9.0 g
		4.52	58.2 a
		9.05	26.0 c
		13.57	16.1 e
		18.10	9.0 g
Dark	4.52		20.1 d
	9.05		12.0 f
	13.57		8.0 g
	18.10		0.0 h
		4.52	30.0 c
		9.05	19.0 d
		13.57	8.0 g
		18.10	0.0 h

Values are means from 10 replications. Treatments with the same *letter* are not significantly different as determined by Tukey's method ($P > 0.01$).

TABLE 2

COMPARISON OF BLACK GRAMA PLANT REGENERATION ON MS MEDIUM FROM CALLUSES INDUCED ON 16 AUXIN AND LIGHT TREATMENTS

Incubation conditions	Auxin ($mg l^{-1}$) 2,4-D	Auxin ($mg l^{-1}$) Dicamba	Plants per 500 mg callus (fresh weight)
Light	4.52		11.5 b
	9.05		2.0 d
	13.57		1.0 d
	18.10		1.2 d
		4.52	21.8 a
		9.05	4.1 c
		13.57	2.6 d
		18.10	1.7 d
Dark	4.52		4.1 c
	9.05		1.1 d
	13.57		1.1 d
	18.10		0.0 e
		4.52	6.0 c
		9.05	2.1 d
		13.57	0.5 d
		18.10	0.0 e

Values are means from 12 replications. Treatments with the same *letter* are not significantly different as determined by Tukey's method ($P > 0.01$).

25 cm in several months (Fig. 1*f*). Compact nodular NEC produced embryos in 20 d and secondary embryos were also frequently observed (Fig. 1*d*). This is in contrast to the development of a friable green callus formed in *B. gracilis* by Aguado-Santa Cruz et al. (2001b).

Conclusions

Substantial numbers of plants can be regenerated from a single *Bouteloua eriopoda* shoot cultured on MS medium supplemented with 4.52 μ*M* Dicamba. Somatic embryogenesis is a valuable research tool for studying *B. eriopoda*, an important range grass species that is rapidly diminishing in arid southwestern rangelands. Intended uses will be to study the nature, distribution, and function of indigenous endophytic fungi that are extensively associated with native *B. eriopoda* populations.

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