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# TISSUE CULTURE AND PLANT REGENERATION OF BLUE GRAMA GRASS, *BOUTELOUA GRACILIS* (H.B.K.) LAG. EX STEUD.

GERARDO ARMANDO AGUADO-SANTACRUZ[1]*, JOSÉ LUIS CABRERA-PONCE[2], VÍCTOR OLALDE-PORTUGAL[3], MA. ROSARIO SÁNCHEZ-GONZÁLEZ[3], JUDITH MÁRQUEZ-GUZMÁN[4], AND LUIS HERRERA-ESTRELLA[2]

[1]*Campo Experimental Bajío, INIFAP-SARH, Apdo. Postal 112, Celaya, Gto. 38110, México*
*CINVESTAV-IPN,* [2]*Departamento de Ingeniería Genética de Plantas and*
[3]*Departamento de Biotecnología y Bioquímica, Apdo. Postal 629, Irapuato, Gto. 36500, México*
[4]*Departamento de Biología, Facultad de Ciencias-UNAM, Apdo. Postal 70-356, México, D.F. 04510, México*

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## Summary

As a first step towards applying biotechnology to blue grama, *Bouteloua gracilis* (H.B.K.) Lag. ex Steud., we have developed a regenerable tissue culture system for this grass. Shoot apices were isolated from 3-d-old seedlings and cultured in 15 different growth regulator formulations combining 2,4-dichlorophenoxyacetic acid (2,4-D), Picloram (4-amino-3,5,6-trichloropicolinic acid), $N^6$-benzyladenine (BA) or adenine (6-aminopurine). The highest induction of organogenic callus was obtained with formulations containing 1 mg $l^{-1}$ (4.52 $\mu M$) 2,4-D plus 0.5 mg $l^{-1}$ (2.22 $\mu M$) BA, and 2 mg $l^{-1}$ (8.88 $\mu M$) BA plus 1 mg $l^{-1}$ (4.14 $\mu M$) Picloram with or without 40 mg $l^{-1}$ (296.08 $\mu M$) adenine. Lower frequencies of induction were obtained for embryogenic as compared to organogenic callus. The most efficient treatments for induction of embryogenic callus contained 2 mg $l^{-1}$ (9.05 $\mu M$) 2,4-D combined with 0.25 (1.11 $\mu M$) or 0.50 mg $l^{-1}$ (2.22 $\mu M$) BA, or 1 mg $l^{-1}$ (4.52 $\mu M$) 2,4-D with 0.50 mg $l^{-1}$ (2.22 $\mu M$) BA. Regeneration was achieved in hormone-free Murashige anmd Skoog (MS) medium, half-strength MS medium or MS medium plus 1 mg $l^{-1}$ (1.44 $\mu M$) gibberellic acid. The number of plantlets regenerated per 500 mg callus fresh weight on MS medium ranged from 9 for 2 mg $l^{-1}$ (9.05 $\mu M$) 2,4-D to 62.2 for induction medium containing 2 mg $l^{-1}$ (8.28 $\mu M$) Picloram, 1 mg $l^{-1}$ (4.44 $\mu M$) BA and 40 mg $l^{-1}$ (296.08 $\mu M$) adenine. Regenerated plants grown in soil under greenhouse conditions reached maturity and produced seeds.

*Key words: Bouteloua gracilis*; blue grama; tissue culture; regeneration; somatic embryogenesis; organogenesis.

## Introduction

Blue grama grass, *Bouteloua gracilis* (H.B.K.) Lag. ex Steud., is a $C_4$, drought-tolerant, perennial grass, native to the North American grassland, which extends from southern Canada to central Mexico (Weaver and Clements, 1938; Hitchcock, 1950). Blue grama is a highly cross-fertilized (Snyder and Harlan, 1953) but facultative apomictic grass (Gustafsson, 1946), which spreads vegetatively by tillers, short root stalks (Pool, 1948; Allred, 1950), and under special conditions through stolons (Stubbendieck et al., 1973).

*B. gracilis* is considered a climax component and probably the most important native grass of the Mexican semiarid grassland (De Alba, 1958; Jaramillo, 1986; Orozco, 1993) and the shortgrass prairie of the USA (Gould, 1951; Sims et al., 1973; Wilson and Briske, 1979). In these communities blue grama can account for 75–90% of the net primary production (Coffin and Lauenroth, 1992) and yields high-quality forage for domestic livestock and native fauna (Stubbendieck et al., 1986).

Plant tissue culture and regeneration systems are central to biotechnological improvement of plants. Primarily applied to major crop species, these technologies have been extended to other species such as forage and turf grasses (Chai and Sticklen, 1998). Despite the economic and ecological importance of blue grama grass, no system for *in vitro* propagation and/or regeneration has been described to date. The objective of this research was to test different growth regulator formulations for the induction of morphogenic responses in shoot apex explants of *Bouteloua gracilis* and to develop a long-term regenerable tissue culture system suitable for its biotechnological improvement. To the best of our knowledge this is the first report on regeneration of plants from tissue culture in blue grama grass.

## Materials and Methods

*Plant material.* Spikes of blue grama used in this study were collected from July to September 1993 in a semiarid grassland located within a region known as 'Los Llanos de Ojuelos', in the north-east of the Jalisco state in central Mexico. Palea and lemma of the spikelets were removed under a stereomicroscope using a pair of dissection needles. Caryopses were surface-sterilized using 70% ethanol for 5 min and 0.02% $HgCl_2$ for 30 min followed by three rinses in sterile distilled water. Using this method, we

*To whom correspondence should be addressed: Gerardo Armando Aguado-Santacruz, Campo Experimental Bajío, INIFAP-SARH, Apdo. Postal 112, Celaya, Gto. 38110, México; Email gaguado@prodigy.net.mx

TABLE 1

GROWTH REGULATOR FORMULATIONS USED FOR INDUCTION OF MORPHOGENIC RESPONSES IN *BOUTELOUA GRACILIS* (H.B.K.) LAG. EX STEUD.

| Formulation | Auxin (mg $l^{-1}$) | | Cytokinin (mg $l^{-1}$) | Additives (mg $l^{-1}$) |
|---|---|---|---|---|
| | 2,4-D | Picloram | BA | Adenine |
| 1 | 1 (4.52 $\mu M$) | | | |
| 2 | 2 (9.05 $\mu M$) | | | |
| 3 | 1 (4.52 $\mu M$) | | 0.25 (1.11 $\mu M$) | |
| 4 | 1 (4.52 $\mu M$) | | 0.50 (2.22 $\mu M$) | |
| 5 | 2 (9.05 $\mu M$) | | 0.25 (1.11 $\mu M$) | |
| 6 | 2 (9.05 $\mu M$) | | 0.50 (2.22 $\mu M$) | |
| 7 | 1 (4.52 $\mu M$) | | 2 (8.88 $\mu M$) | 20 (148.04 $\mu M$) |
| 8 | 1 (4.52 $\mu M$) | | 2 (8.88 $\mu M$) | 40 (296.08 $\mu M$)[a] |
| 9 | 1 (4.52 $\mu M$) | | 2 (8.88 $\mu M$) | 80 (592.15 $\mu M$) |
| 10 | | 2 (8.28 $\mu M$) | 1 (4.44 $\mu M$) | |
| 11 | | 2 (8.28 $\mu M$) | 1 (4.44 $\mu M$) | 40 (296.08 $\mu M$) |
| 12 | | 2 (8.28 $\mu M$) | 1 (4.44 $\mu M$) | 80 (592.15 $\mu M$) |
| 13 | | 1 (4.14 $\mu M$) | 2 (8.88 $\mu M$) | |
| 14 | | 1 (4.14 $\mu M$) | 2 (8.88 $\mu M$) | 40 (296.08 $\mu M$) |
| 15 | | 1 (4.14 $\mu M$) | 2 (8.88 $\mu M$) | 80 (592.15 $\mu M$) |

[a] MPC medium (J. L. Cabrera-Ponce, personal communication).

obtained 70% germination in 9 g $l^{-1}$ solidified agar. Compared to $HgCl_2$, commercial bleach was an ineffective sterilizing agent. Initial experiments employed mature caryopses as explants, but abnormalities in seedlings or non-germinating caryopses lowered viable explant number. Therefore, in further experiments we used the shoot apices of dark-germinated 3-d-old seedlings as the starting material. We isolated the shoot apices by cutting 2 mm under and above the coleoptile node of the seedlings.

*Callus induction and maintenance.* A total of 15 growth regulator formulations including the auxins 2,4-dichlorophenoxyacetic acid (2,4-D) or Picloram (4-amino-3,5,6-trichloropicolinic acid), the cytokinin $N^6$-benzyladenine (BA), or adenine (6-aminopurine), were evaluated for induction of morphogenic callus (Table 1). All formulations included MS basal medium (Murashige and Skoog, 1962; Gibco, Frederick, MD) containing 3% sucrose and 3 g $l^{-1}$ phytagel (Sigma, St Louis, MO) (pH 5.8, autoclaved at 120°C for 15 min). For each treatment 110 shoot apices were cultured (10 apices per Petri dish). Explants were maintained in darkness for 76 d (25°C) from the initial culture and subcultured every 25 d on the same induction media. Induction efficiency of organogenic and embryogenic calluses (number of morphogenic calluses/total number of explants × 100) was evaluated every 19 d from the initial culture of apices to day 76.

*Plant regeneration.* To promote regeneration, calluses were transferred to hormone-free, half-strength MS medium, or full-strength MS medium with or without 1 mg $l^{-1}$ gibberellic acid, and incubated under fluorescent light (117 $\mu$mol $s^{-1}$ $m^{-2}$; 16 h photoperiod) at 25°C. A comparative analysis of the regeneration capacity among the 15 evaluated treatments was carried out by transferring 500 mg (fresh weight) of the fastest growing callus from each treatment, independently of its morphogenic nature, to hormone-free MS medium. One-way analysis of variance was used to test significant differences in the regeneration efficiency between treatments. Means were separated using Tukey's test (Zar, 1974). When plantlets developed four to eight tillers, they were transferred to baby-food jars containing autoclaved vermiculite (5 g) and half-strength liquid MS medium (28 ml; pH 5.8) for 1 mo. and then transplanted into pots containing a sterilized mixture of sand with a fine-textured soil and transferred to the greenhouse.

## Results

*Callus induction.* As starting material, we used the apical shoot apex of 3-d-old seedlings. To identify and isolate this explant, seeds were germinated in the dark to promote elongation of the subcoleoptile internode, which in grasses is controlled by the phytochrome system (Roohi et al., 1991; Tischler et al., 1997).

After 57 d in culture, morphogenic responses from shoot apex-derived calluses were observed at low frequencies in all 15 different growth regulator formulations tested (Tables 2 and 3). Organogenic callus was pale yellow to light green in color with numerous small green shoots (Fig. 1a), while embryogenic callus was pale yellow to light beige. An exception to this type of embryogenic callus was found in MPC treatment (2 mg $l^{-1}$ BA + 1 mg $l^{-1}$ 2,4-D + 40 mg $l^{-1}$ adenine; J. L. Cabrera-Ponce, personal communication), in which a very friable green callus was obtained. After transferring this callus to liquid MPC, we were successful in obtaining a highly chlorophyllous embryogenic cell suspension (Aguado-Santacruz et al., 2000).

The presence of a discrete and closed vascular system and a bipolar structure were used as criteria for distinguishing embryogenic from organogenic calluses (Fig. 1b). Additionally, embryogenic callus was commonly immersed within a mucilaginous matrix, similar to that described by Ahn et al. (1987) for Bermudagrass (*Cynodon dactylon*). Embryogenic callus developed a clear globular phase (Fig. 1c) and in some treatments acquired a hairy appearance when transferred to regeneration medium (Fig. 1d). Further light microscopy analysis confirmed our previous observations on the morphogenic nature of the calluses. Proembryogenic cells immersed within vacuolated cell masses were evident in embryogenic callus. A bipolar structure with vascular connection in developing embryos was also noticed (Fig. 2).

The morphogenic nature of the calluses was not exclusive, since embryogenic callus sometimes showed small sectors of organogenic tissue. Embryogenic calluses developing small sectors of shoots via organogenesis were scored as embryogenic in the assessment of the induction efficiency of morphogenic responses. Embryogenic callus was always maintained in darkness prior to regeneration tests since spontaneous germination commonly took place when exposed to light.

The maximum frequencies of induction of organogenic and embryogenic calluses were obtained 57 d after initial culture of explants (Tables 2 and 3) in all treatments, except in that containing

TABLE 2

INDUCTION EFFICIENCY OF ORGANOGENIC CALLUS (%)[a] IN 15 DIFFERENT GROWTH REGULATOR FORMULATIONS IN *BOUTELOUA GRACILIS* (H.B.K.) LAG. EX STEUD. AT FOUR DATES AFTER INITIAL CULTURE OF EXPLANTS

| | Days after initial culture | | | |
|---|---|---|---|---|
| Formulations | 19 | 38 | 57 | 76 |
| 2,4-D 1 mg $l^{-1}$ | 0 | 1.0 | 1.0 | 1.0 |
| 2,4-D 2 mg $l^{-1}$ | 0 | 0 | 1.0 | 1.0 |
| 2,4-D 1 mg $l^{-1}$ + BA 0.25 mg $l^{-1}$ | 0 | 2.0 | 2.0 | 2.0 |
| 2,4-D 1 mg $l^{-1}$ + BA 0.50 mg $l^{-1}$ | 0 | 7.3 | 7.3 | 7.3 |
| 2,4-D 2 mg $l^{-1}$ + BA 0.25 mg $l^{-1}$ | 0 | 1.1 | 1.1 | 1.1 |
| 2,4-D 2 mg $l^{-1}$ + BA 0.50 mg $l^{-1}$ | 0 | 0 | 0 | 0 |
| BA 2 mg $l^{-1}$ + 2,4-D 1 mg $l^{-1}$ + adenine 20 mg $l^{-1}$ | 0 | 2.8 | 2.8 | 2.8 |
| BA 2 mg $l^{-1}$ + 2,4-D 1 mg $l^{-1}$ + adenine 40 mg $l^{-1}$ | 1.0 | 2.2 | 2.2 | 2.2 |
| BA 2 mg $l^{-1}$ + 2,4-D 1 mg $l^{-1}$ + adenine 80 mg $l^{-1}$ | 1.9 | 1.9 | 2.8 | 2.8 |
| Picloram 2 mg $l^{-1}$ + BA 1 mg $l^{-1}$ | 0.9 | 0.9 | 0.9 | 0.9 |
| Picloram 2 mg $l^{-1}$ + BA 1 mg $l^{-1}$ + adenine 40 mg $l^{-1}$ | 0 | 2.1 | 2.1 | 2.1 |
| Picloram 2 mg $l^{-1}$ + BA 1 mg $l^{-1}$ + adenine 80 mg $l^{-1}$ | 0.9 | 0.9 | 0.9 | 0.9 |
| BA 2 mg $l^{-1}$ + Picloram 1 mg $l^{-1}$ | 2.7 | 4.5 | 4.5 | 4.5 |
| BA 2 mg $l^{-1}$ + Picloram 1 mg $l^{-1}$ + adenine 40 mg $l^{-1}$ | 3.7 | 4.7 | 4.7 | 4.7 |
| BA 2 mg $l^{-1}$ + Picloram 1 mg $l^{-1}$ + adenine 80 mg $l^{-1}$ | 0 | 4.1 | 4.1 | 4.1 |

[a] Number of organogenic calluses/total number of explants ×100.

TABLE 3

INDUCTION EFFICIENCY OF EMBRYOGENIC CALLUS (%)[a] IN 15 DIFFERENT GROWTH REGULATOR FORMULATIONS IN *BOUTELOUA GRACILIS* (H.B.K.) LAG. EX STEUD. AT FOUR DATES AFTER INITIAL CULTURE OF EXPLANTS

| | Days after initial culture | | | |
|---|---|---|---|---|
| Formulations | 19 | 38 | 57 | 76 |
| 2,4-D 1 mg $l^{-1}$ | 0 | 0 | 0 | 0 |
| 2,4-D 2 mg $l^{-1}$ | 0 | 0 | 0 | 0 |
| 2,4-D 1 mg $l^{-1}$ + BA 0.25 mg $l^{-1}$ | 0 | 1.0 | 1.0 | 1.0 |
| 2,4-D 1 mg $l^{-1}$ + BA 0.50 mg $l^{-1}$ | 0 | 1.0 | 1.0 | 2.1 |
| 2,4-D 2 mg $l^{-1}$ + BA 0.25 mg $l^{-1}$ | 0 | 2.1 | 3.2 | 3.2 |
| 2,4-D 2 mg $l^{-1}$ + BA 0.50 mg $l^{-1}$ | 0 | 2.0 | 2.0 | 2.0 |
| BA 2 mg $l^{-1}$ + 2,4-D 1 mg $l^{-1}$ + adenine 20 mg $l^{-1}$ | 0 | 0 | 0 | 0 |
| BA 2 mg $l^{-1}$ + 2,4-D 1 mg $l^{-1}$ + adenine 40 mg $l^{-1}$ | 1.1 | 1.1 | 1.1 | 1.1 |
| BA 2 mg $l^{-1}$ + 2,4-D 1 mg $l^{-1}$ + adenine 80 mg $l^{-1}$ | 0.9 | 1.9 | 1.9 | 1.9 |
| Picloram 2 mg $l^{-1}$ + BA 1 mg $l^{-1}$ | 0.9 | 0.9 | 0.9 | 0.9 |
| Picloram 2 mg $l^{-1}$ + BA 1 mg $l^{-1}$ + adenine 40 mg $l^{-1}$ | 0 | 0 | 0 | 0 |
| Picloram 2 mg $l^{-1}$ + BA 1 mg $l^{-1}$ + adenine 80 mg $l^{-1}$ | 0.9 | 1.9 | 1.9 | 1.9 |
| BA 2 mg $l^{-1}$ + Picloram 1 mg $l^{-1}$ | 0 | 1.8 | 1.8 | 1.8 |
| BA 2 mg $l^{-1}$ + Picloram 1 mg $l^{-1}$ + adenine 40 mg $l^{-1}$ | 0 | 0 | 0 | 0 |
| BA 2 mg $l^{-1}$ + Picloram 1 mg $l^{-1}$ + adenine 80 mg $l^{-1}$ | 0 | 0 | 0 | 0 |

[a] Number of embryogenic calluses/total number of explants ×100.

1 mg $l^{-1}$ 2,4-D plus 0.5 mg $l^{-1}$ BA, in which a 2-fold increase in the percentage of embryogenic calluses was observed in the last evaluation, i.e. by day 76 (Table 3). A faster induction of morphogenic responses was obtained in treatments containing 2 mg $l^{-1}$ BA plus 1 mg $l^{-1}$ 2,4-D with addition of 40 or 80 mg $l^{-1}$ adenine, 2 mg $l^{-1}$ Picloram plus 1 mg $l^{-1}$ BA with or without addition of 80 mg $l^{-1}$ adenine, or 2 mg $l^{-1}$ BA plus 1 mg $l^{-1}$ Picloram with or without addition of 40 mg $l^{-1}$ adenine (Tables 2 and 3).

Formulations containing 1 mg $l^{-1}$ 2,4-D plus 0.5 mg $l^{-1}$ BA, or 2 mg $l^{-1}$ BA plus 1 mg $l^{-1}$ Picloram with or without addition of 40 mg $l^{-1}$ adenine produced the highest induction of organogenic calluses with 7.3, 4.7 and 4.5%, respectively (Table 2). Lower frequencies of induction were obtained for embryogenic as compared to organogenic calluses (Table 3). The most efficient treatments for induction of embryogenic calluses contained 2 mg $l^{-1}$ 2,4-D combined with 0.25 (3.2%) or 0.50 mg $l^{-1}$ BA (2.0%), and 2,4-D at 1 mg $l^{-1}$ with 0.50 mg $l^{-1}$ BA (2.1%; Table 3).

*Plant regeneration.* After four subcultures (carried out every 25 d) on the original induction media, organogenic and embryogenic calluses were transferred to hormone-free half-strength MS medium, full-strength MS medium and full-strength MS medium plus 1 mg $l^{-1}$ gibberellic acid to induce regeneration (Fig. 1e). Similar efficiencies of regeneration were observed in the three media tested (data not shown). Embryogenic callus developed complete plantlets after 45–50 d, while shoots derived via organogenesis produced roots 8–14 d after transfer to regeneration

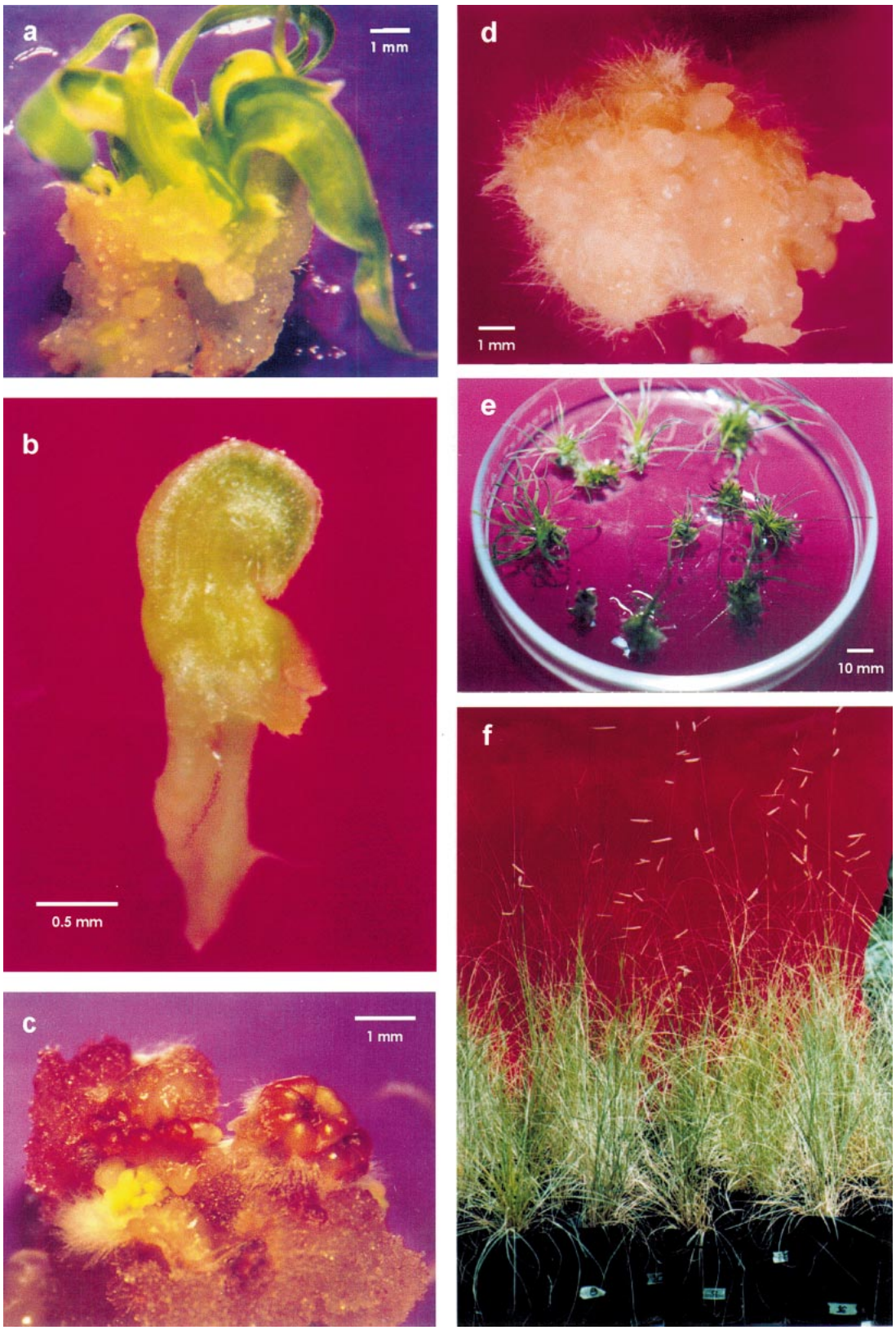


FIG. 1. *In vitro* culture and regeneration of *Bouteloua gracilis*. a, Organogenic callus showing multiple shoots. b, Germinated embryo 20 d after transfer to MS medium without growth regulators showing a bipolar structure. c, Globular phase in an embryogenic callus. d, Hairy appearance in a regenerating embryogenic culture 12 d after transfer to MS medium without growth regulators. e, Regeneration from a single embryogenic callus induced on 2 mg $l^{-1}$ 2,4-D plus 0.25 mg $l^{-1}$ BA. Plantlets developed 45 d after transfer to MS medium without growth regulators. f, Seed setting in a greenhouse in plants regenerated from tissue culture 6 mo. after transplanting to soil.

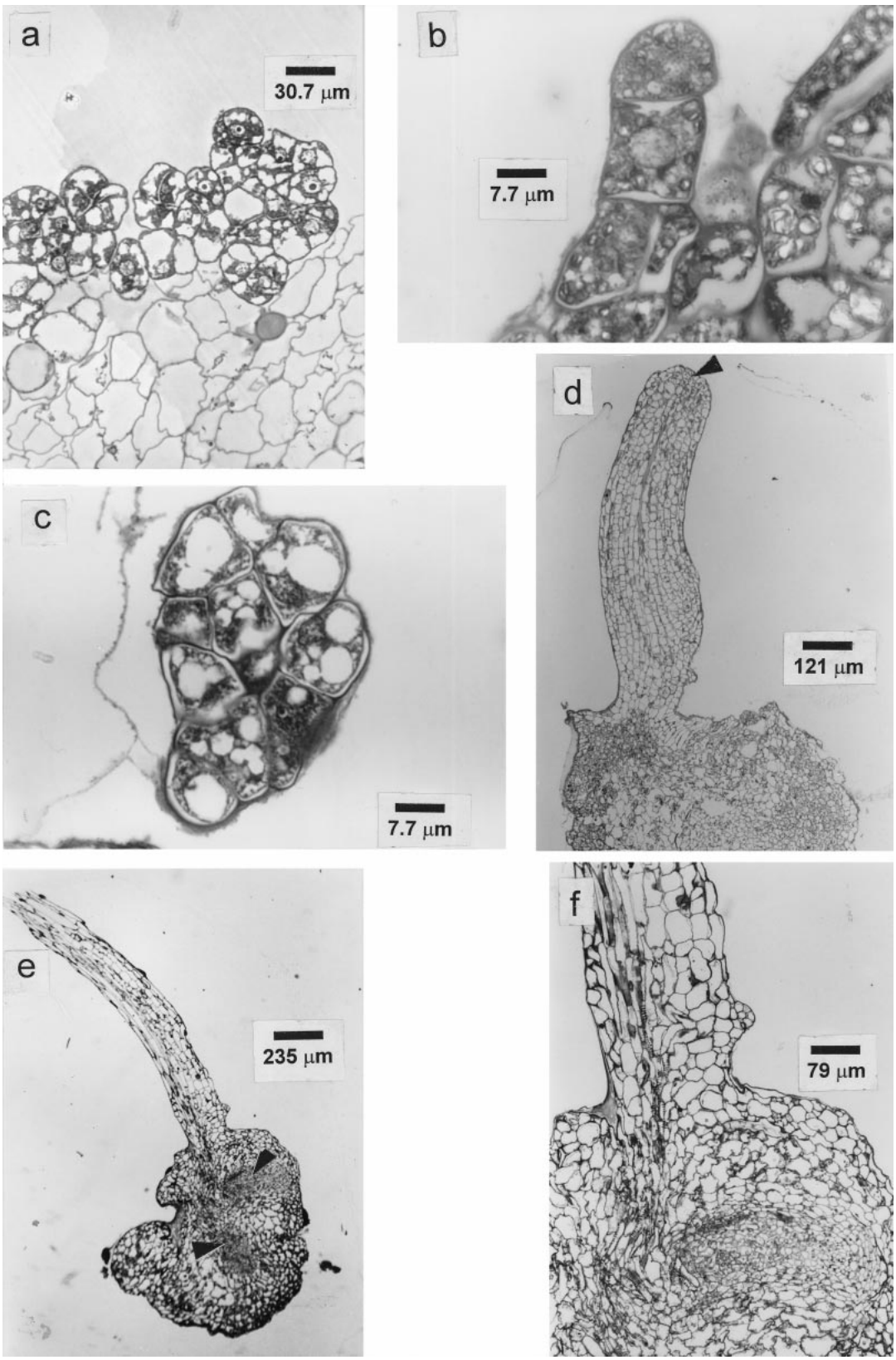


FIG. 2. Somatic embryogenesis in *Bouteloua gracilis*. a, Small, dense cytoplasmic proembryogenic cells with very prominent nuclei and starch grains. b, A dividing four-celled proembryo. c, Pluricellular proembryo. d, Embryo showing a well-developed epidermis and a region of active division at the apical pole. e, Bipolar embryo showing two radicle initials. f, Shoot-radicle vascular connection.

TABLE 4

*IN VITRO* PLANT REGENERATION OF *BOUTELOUA GRACILIS* (H.B.K.) LAG. EX STEUD. ON MS MEDIUM FROM CALLUSES INDUCED ON 15 DIFFERENT TREATMENTS

| Auxin (mg $l^{-1}$) | | Cytokinin (mg $l^{-1}$) | Additives (mg $l^{-1}$) | | |
|---|---|---|---|---|---|
| 2,4-D | Picloram | BA | Adenine | Plantlets per 500 mg callus (fresh weight)[a] | |
| 1 | | | | 9.0 | h |
| 2 | | | | 12.5 | gh |
| 1 | | 0.25 | | 35.5 | b |
| 1 | | 0.50 | | 30.5 | cd |
| 2 | | 0.25 | | 37.2 | b |
| 2 | | 0.50 | | 34.5 | bc |
| 1 | | 2 | 20 | 14.0 | fg |
| 1 | | 2 | 40 | 13.5 | fg |
| 1 | | 2 | 80 | 17.2 | f |
| | 2 | 1 | | 23.5 | e |
| | 2 | 1 | 40 | 62.2 | a |
| | 2 | 1 | 80 | 27.6 | de |
| | 1 | 2 | | 13.3 | fg |
| | 1 | 2 | 40 | 14.7 | fg |
| | 1 | 2 | 80 | 15.8 | fg |

[a] Average values from 10 replications. Treatments with the same letter are not significantly different as determined by Tukey's mean separation ($P \geq 0.05$).

media. Albino shoots were sporadically observed in regenerating organogenic callus but never from embryogenic callus.

When transferred to full-strength MS medium, calluses initiated on 2 mg $l^{-1}$ Picloram plus 1 mg $l^{-1}$ BA and 40 mg $l^{-1}$ adenine produced a statistically superior number of plantlets (62.2) per 500 mg (FW) callus (Table 4). The number of plantlets regenerated in treatments combining 1 or 2 mg $l^{-1}$ 2,4-D with 0.25 or 0.50 mg $l^{-1}$ BA ranged from 30.5 to 37.2, while treatments including 1 or 2 mg $l^{-1}$ 2,4-D alone, and those combining Picloram or 2,4-D at 1 mg $l^{-1}$ with BA 2 mg $l^{-1}$ with or without adenine added, resulted in a low number of regenerated plantlets (Table 4). The fastest growing organogenic and embryogenic cell lines of *Bouteloua gracilis* were selected and maintained by subculturing on the original induction media. After 1 yr in culture, the regeneration capacity was retained in these fast-growing morphogenic lines. Meticulous selection of callus, avoiding non-morphogenic, root-forming and/or albino-producing white organogenic calluses, has been an essential procedure for successful conservation of this characteristic.

Vigorous plants with a profuse root system resulted from transferring four to eight tiller plantlets to baby-food jars containing vermiculite and half-strength MS liquid medium for 1 mo. Most plantlets transplanted to pots with a mixture of sand and soil survived. From the initial induction of callus, 5–6 mo. were required to produce plantlets with the potential to be established in soil, and 12 mo. to produce seeds under greenhouse conditions (Fig. 1f). We were able to establish up to 100 plants in the greenhouse from callus induced in treatment containing 2 mg $l^{-1}$ Picloram, 1 mg $l^{-1}$ BA and 40 mg $l^{-1}$ adenine, and 75 with that including 2 mg $l^{-1}$ 2,4-D and 0.25 mg $l^{-1}$ BA. Similar efficiencies of plantlet establishment in the greenhouse were observed among treatments (data not shown), and it depended more on the presence of a well-developed root system than on the growth regulator formulation used for callus induction or on the morphogenic pathway through which plantlets were regenerated.

## Discussion

The previously described recalcitrance of grasses to *in vitro* manipulation has been overcome by considering primary factors affecting tissue culture such as media constituents, explant source, genotype, and physical and chemical conditions of culture (Vasil, 1987). Regeneration systems have now been developed for many grass species including forage (Chen et al., 1977; Lo et al., 1980; Bajaj et al., 1981; Songstad, 1983; Johnson and Worthington, 1987; Metzinger et al., 1987; Straub et al., 1989; Franklin et al., 1990; Akashi and Adachi, 1992) and turf (Lee, 1996) to ornamental (Robacker and Corley, 1992) and biofuel grasses (Denchev and Conger, 1994). Here we report the first protocol for inducing embryogenic and organogenic calluses in the forage grass *Bouteloua gracilis* using the shoot apex as starting material. Previously, Riordan et al. (1997) reported the technology for tissue culture of buffalo grass (*Buchloë dactyloides*), also a component of the shortgrass prairie.

Different sources for callus induction in grasses have been used: mature caryopses and immature embryos, immature inflorescences, mesocotyl plate tissue and leaf-stem sections of stolons. The shoot apex has been successfully used as explant in cereals and millets, but less frequently in forage grass tissue culture (Bajaj et al., 1981).

The small size of *Bouteloua gracilis* caryopses prevents isolation of embryos and their use as explants, while the abnormal development of seedlings encountered in this study makes complete caryopses of this grass also unsuitable for initiation of tissue culture systems. Van der Valk et al. (1989) found that callus induction from seeds of Kentucky bluegrass (*Poa pratensis*) was strongly related with seed germination. Thus, testing of alternative sources of explants and genotypes in *Bouteloua gracilis* will be important in the future.

Although 2,4-D has been the most commonly used auxin in cereal tissue culture (Bhaskaran and Smith, 1990), other auxins such as Picloram and Dicamba have been found to be more effective

for induction of morphogenic responses in some grasses (Conger et al., 1982; McDonnell and Conger, 1984; Zhong et al., 1991). In our study we found a superior response when auxins were used in combination with low concentrations of cytokinins. The formulation containing 1 mg $l^{-1}$ 2,4-D plus 0.50 mg $l^{-1}$ BA appeared to be adequate for induction of both organogenic and embryogenic calluses, however, the combination of 2 mg $l^{-1}$ Picloram with 1 mg $l^{-1}$ BA and 40 mg $l^{-1}$ adenine gave the best regenerative response via organogenesis.

Induction frequencies of morphogenic callus in this study were low (7.3 calluses per 100 shoot apices in the best case). Similar percentages were reported by other authors. For example, Akashi and Adachi (1992) found a 4.3% induction of embryogenic callus in the cultivar Louisiana B-230 of *Paspalum dilatatum* using 5 mg $l^{-1}$ 2,4-D. Boyd and Dale (1986) obtained variable frequencies of organogenic and embryogenic calluses in cultivars 'Merion' and 'Victa' of *Poa pratensis* as a function of varying 2,4-D, sucrose and coconut milk concentrations; induction percentages ranged from 0 to 61% for organogenic, and 0 to 36% for embryogenic calluses. Ahn et al. (1987) could not promote somatic embryogenesis in three of seven materials of Bermudagrass (*Cynodon dactylon*) tested, while 7% induction was attained in cultivar 'Tifton 44'. In creeping bentgrass (*Agrostis palustris*), Zhong et al. (1991) used 2,4-D at concentrations of 2.25, 4.5, 9.0 and 18 $\mu M$ achieving frequencies of 0, 3.8, 20 and 3.2% of embryogenic calluses formation, respectively.

Despite the low efficiencies for organogenic and embryogenic callus induction reported in this work, once the desired morphogenic response was attained, calluses produced with formulations containing 2 mg $l^{-1}$ Picloram plus 1 mg $l^{-1}$ BA and 40 mg $l^{-1}$ adenine, and those combining 1 or 2 mg $l^{-1}$ 2,4-D with 0.25 or 0.50 mg $l^{-1}$ BA, were capable of generating morphogenic lines with abundant material in a short time (Fig. 1e).

Although gibberellic acid at concentration 1 mg $l^{-1}$ has been used to promote the germination of somatic embryos in maize (Lu et al., 1982), we did not find any improvement in the regeneration efficiency of morphogenic callus of *B. gracilis* using this hormone. However, a better rooting in plantlets is predicted when used at 0.5 mg $l^{-1}$ and combined with 1 mg $l^{-1}$ indole-3-acetic acid (Roohi and Jameson, 1991). Studies are now being conducted to test it.

This work sets a starting point for developing more efficient protocols for tissue culture of *Bouteloua gracilis* for use in biotechnological management of this important forage grass of the North American grassland. Of special interest will be determining the amenability of the faster growing morphogenic callus lines generated in this study to the diverse methodologies now available for direct gene transfer. This would permit the evaluation of genes with capabilities for altering the structure and/or physiology of plants to solve an old and well-documented problem in *Bouteloua gracilis*, namely the establishment of seedlings under natural conditions (Hyder et al., 1971). Also important would be the improvement of the blue grama forage quality, a topic gaining relevance in forage plants (Tabe et al., 1995; Heath et al., 1998). This would be possible by genetic transformation of the materials evaluated in this work or by crossing them with superior cultivars of *Bouteloua gracilis* such as 'Alma', 'Lovington' and 'Hachita', which are all varieties released primarily by the Plant Materials Center-USDA-NRCS of New Mexico.

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