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Establishment, characterization and plant regeneration from highly chlorophyllous embryogenic cell cultures of blue grama grass, *Bouteloua gracilis* (H.B.K.) Lag. ex Steud.

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Abstract A finely dispersed, homogeneous and highly chlorophyllous cell suspension (TIANSJ98 cell line) was obtained from shoot apices of *Bouteloua gracilis* (H.B.K.) Lag. ex Steud. cultured on MPC medium containing MS salts supplemented with 2,4-D (1 mg/l), BAP (2 mg/l) and adenine (40 mg/l). When the TIANSJ98 cell line was grown in this medium with shaking at 180 rpm it had doubling times of 7.2 and 3.7 days in terms of fresh and dry weight, respectively. Total chlorophyll content in this cell culture ranged from 121.6 to 18.3 μg/g FW at 12 and 21 days following culture initiation. Plants regenerated from the TIANSJ98 cell line, via somatic embryogenesis, were grown to maturity and produced seeds. Although different cell culture systems have been described for cereals and grasses, to the best of our knowledge this is the first report of a highly chlorophyllous and regenerable cell suspension in Poaceae.

Keywords *Bouteloua gracilis* · Cell culture · Somatic embryogenesis · Chlorophyllous · Photoautotrophy

Abbreviations *BAP* N^6–Benzylaminopurine · *2,4-D* 2,4-Dichlorophenoxyacetic acid · *FW* Fresh weight · *MS* Murashige and Skoog (l962)

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Introduction

Plant cell cultures represent unique systems for the controlled analysis of physiological, biochemical, cytological and genetic processes of higher plants at the cellular level (Lerner 1985; Ziegler and Scheibe 1989; Widholm 1992). Chlorophyllous cell cultures, in which stomata are absent and certain enzymes and metabolic pathways are confined to photosynthetically active plastids (Widholm 1992), offer additional advantages, especially when grown photoautotrophically, the latter representing potentially more realistic (Roeske et al. 1989) and less complicated models (Avelange et al. 1991) for studying photosynthesis, analyzing the effects of osmotic, cold or heat stresses on photosynthesis and chloroplast development (Widholm 1992), while allowing the production of specific secondary metabolites (Fischer et al. 1994). Recently we developed the first tissue culture and regeneration system for blue grama grass, *Bouteloua gracilis* (H.B.K.) Lag. ex Steud. (Aguado-Santacruz et al. 2000), which is a facultative apomictic, C_4-photosynthetic, drought-tolerant, warm-season perennial and important forage grass of the semiarid regions of the United States and Mexico (Gustafsson 1946; Hoover et al. 1948; Walker and Lewis 1979), where it yields abundant and high-quality forage for livestock and native fauna (Stubbendieck et al. 1986; Coffin and Lauenroth 1992). Due to its economical importance, drought-resistance characteristics and wide distribution this grass is the subject of intensive ecological and physiological research (Majerus 1975; Brown and Trlica 1977; Monson et al. 1986; Aguilera and Lauenroth 1993; Bowman and Turner 1993; Dodd et al. 1998; Hunt et al. 1998; Morgan et al. 1998).

We describe here the development of a regenerable chlorophyllous cell suspension of *B. gracilis* (named TIANSJ98 cell line). Some characteristics and potential applications of this chlorophyllous cell line are discussed.

Materials and methods

Callus initiation

Shoot apices of *Bouteloua gracilis* (H.B.K.) Lag ex. Steud. were isolated from 110 3-day-old seedlings as previously described (Aguado-Santacruz et al. 2000) and cultured in the dark (10 apices per petri dish) on solidified MPC medium (J.L. Cabrera-Ponce, personal communication) containing MS basal medium (Gibco) supplemented with 2,4-D (1 mg/l), BAP (2 mg/l), adenine (40 mg/l), sucrose (3%) and phytagel (3 g/l; Sigma). The pH of the medium was adjusted to 5.8 before autoclaving (120 °C for 15 min). Calli were subcultured every 25 days on the same induction medium. Half of the callus produced by each explant was transferred to light conditions 38 days after initiation of the culture (fluorescent lamps, 117 μmol s^{-1} m^{-2}), 25° ± 1 °C, and the other half of the material was maintained in the dark (25° ± 1 °C).

Cell culture initiation and characterization

After 2 months of growth under light conditions, a highly friable green callus developed from one explant, which was then transferred to liquid MPC medium and maintained on a gyratory shaker at 70 rpm under continuous fluorescent lighting (60 μmol s^{-1} m^{-2}), 30° ± 1 °C. A finely dispersed, homogeneous and chlorophyllous cell suspension was obtained after 3 weeks of shaking. The efficiency of growth of this cell line was determined under liquid and solid conditions. For evaluation of the weight gain on solid MPC medium, we placed 0.07 g from an exponentially growing cell culture on filter paper on the solid medium and replaced the medium weekly. This material was incubated in a growth chamber at 25° ± 1 °C under a light intensity of 117 μmol s^{-1} m^{-2}. Fresh weight increase was determined every 7 days for 35 days. Conversely, growth efficiency in liquid MPC was evaluated by batch culture. Forty-eight 125-ml flasks containing 24 ml of liquid MPC medium were inoculated with 1 ml of the cell suspension (0.5 g) from a 13-day-old culture and agitated at 180 rpm on a gyratory shaker at 30° ± 1 C under continuous fluorescent lighting (60 μmol s^{-1} m^{-2}). Fresh and dry weights were destructively determined every 3 days for 21 days. After weighing, three of the samples were used for determination of chlorophyll content (total, *a* and *b*) at each sampling date. Chlorophyll was extracted from cells with aqueous acetone (80% v/v) and determined according to the procedure of Arnon (1949).

Regeneration of plants

The regenerative potential of the TIANSJ98 cell suspension was tested by transferring 1 g of an exponentially growing culture onto solidified MS medium amended with 6% sucrose or MS medium containing either 3% sucrose alone or supplemented with abscisic acid, gibberellic acid, 2,4-D, kinetin or BAP (0.05, 0.10, 0.25, 0.50 and 1 mg/l) or adenine (10, 20 and 40 mg/l). The petri dishes were placed at 30° ± 1 °C under continuous fluorescent lighting (60 μmol s^{-1} m^{-2}).

Results and discussion

Initiation of the cell suspension

Organogenic and embryogenic calli were obtained from shoot apices cultured on MPC medium after 38 days of culture at a ratio of 2/91 and 1/91 (no. of morphogenic calli obtained/no. of explants cultured), respectively. Organogenic calli were very lignified, while embryogenic calli showed a clear globular phase (Aguado-Santacruz et al. 2000). After identification and isolation of these morphogenic calli, a second subculture was carried out and part of the plant material transferred to light. Two months later a section of a callus, originally rated as organogenic developed a highly friable green callus (Fig. 1a), which was then placed in liquid MPC medium. This callus probably consisted of a mixture of organogenic and embryogenic clusters, a situation observed previously in *B. gracilis* tissue culture (Aguado-Santacruz et al. 2000). A finely dispersed, homogeneous and chlorophyllous cell suspension (named TIANSJ98 cell line) was obtained after 3 weeks of growth under continuous fluorescent lighting (Fig. 1b). The establishment of the cell suspensions from the green calli could be repeatedly achieved when required for at least 1 year. Although this finely dispersed cell suspension tended to form aggregates, it could be subcultured routinely every 20 days by transferring 1 ml of the cell suspension into 24 ml of fresh medium using a 1-ml pipettor fitted to 1-mm-diameter tips. Different morphologies were observed in the TIANSJ98 cell suspension. Round cells had an average diameter ranging between 25 μm and 35 μm (Fig. 1c, d) and showed apparently well-developed chloroplasts (Fig. 1e). Tubular and aggregated cells similar to those described by Ahn et al. (1987) for Bermuda grass (*Cynodon dactylon*) were also present. Although different cell culture systems have been described for cereals (Bhaskaran and Smith 1990) and grasses (Ahloowalia 1984; Chai and Sticklen 1998), to the best of our knowledge this is the first report of a chlorophyllous cell suspension in Poaceae that also possesses regeneration potential. Explanations for this notable absence of reports on chlorophyllous or photoautotrophic cultures in this family include the general lack of uniform greening of cultured cells (Widholm 1992) and the inhibition of chloroplast differentiation due to the high auxin concentration required for callus induction in graminaceous plants (Yamada 1985).

Growth of TIANSJ98 cell suspension

When grown on solid MPC medium that was replaced every 7 days, the TIANSJ98 cell line showed an exponential increase in fresh weight, with a doubling of the initial biomass in 9.8 days (Fig. 2). In batch culture, the cell suspension showed doubling times in biomass of 7.2 days with respect to fresh weight and 3.7 days for dry weight (Fig. 3). When the agitation was increased this variable decreased – growth at 70 rpm produced doubling times of 9.5 and 4.3 days for fresh and dry weight, respectively (data not shown). When the kinetics of cell growth was plotted, a lag phase of about 6 days was evident previous to the phase of exponential increase. The greatest gain in fresh weight was attained at day 18 (fivefold increase), while the maximum 33-fold increase in dry weight was observed by day 15 (Fig. 3).

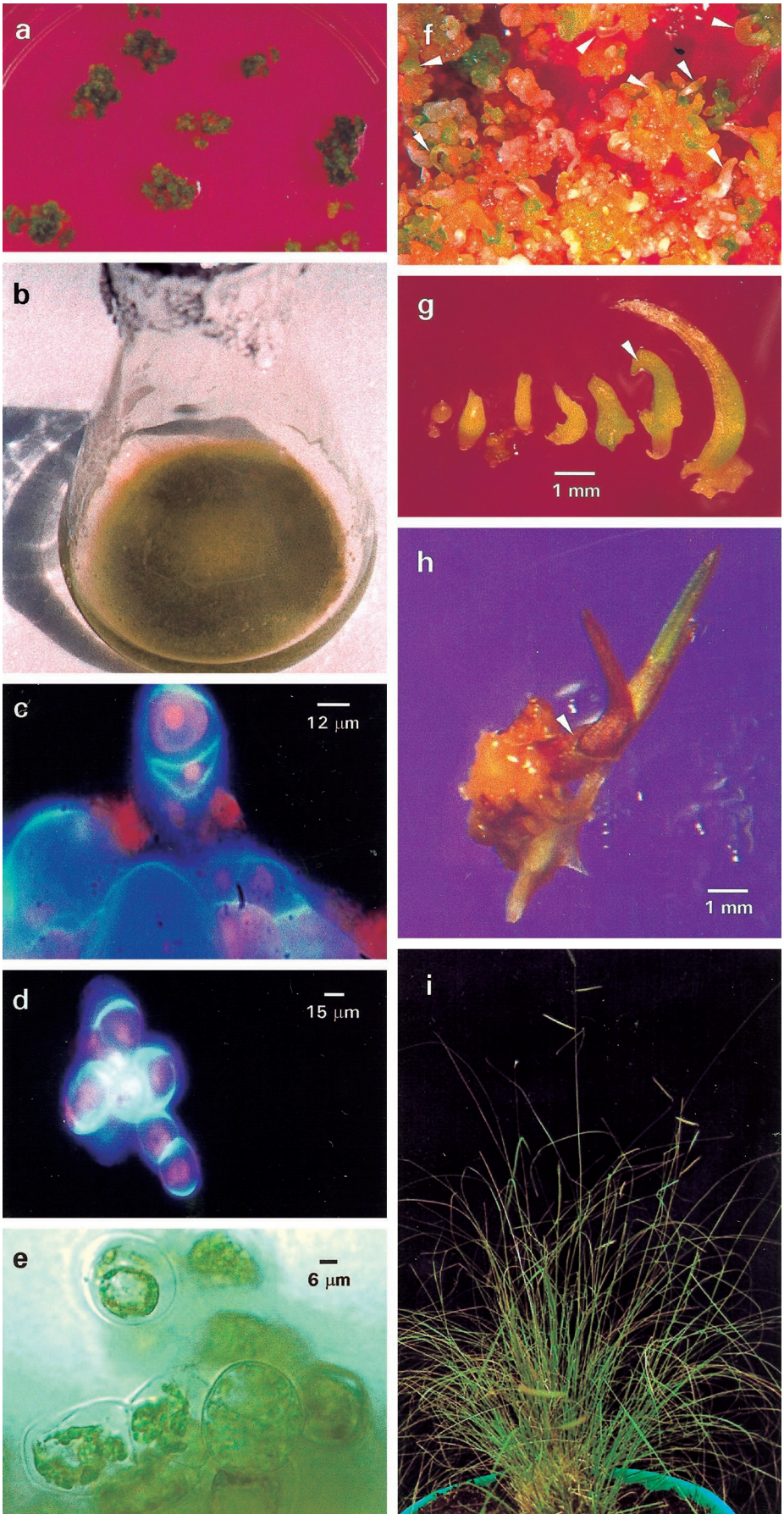

Fig. 1a–i Initiation of the highly chlorophyllous TIANSJ98 cell line and somatic embryogenesis in *Bouteloua gracilis*. **a** Green calli obtained from culturing blue grama shoot apices on MPC medium. b TIANSJ98 chlorophyllous cells after three weekly transfers of 500 mg green callus initial inoculum to fresh MPC medium with 70 rpm shaking. **c** Dividing cell stained with calcofluor white M2R and ethidium bromide. **d** A seven-cell aggregate formed after 9 days of subculture in liquid MPC. **e** TIANSJ98 cells exhibiting well-developed chloroplasts (chlorophyll content: 100 μg/g fresh weight). **f** Multiple regenerating somatic embryos after 1 month of subculturing on MS amended with 6% sucrose. **g** Somatic embryogenesis in chlorophyllous cell cultures of *B. gracilis*; somatic embryos develop very conspicuous coleoptiles (indicated by *arrow*). **h** Plantlet obtained after 48 days of subculture on MS amended with 6% sucrose; remnants of the coleoptile in the developing plantlet are indicated by the *arrow*. **i** Regenerated plant established in the greenhouse showing the characteristic spikes of the species

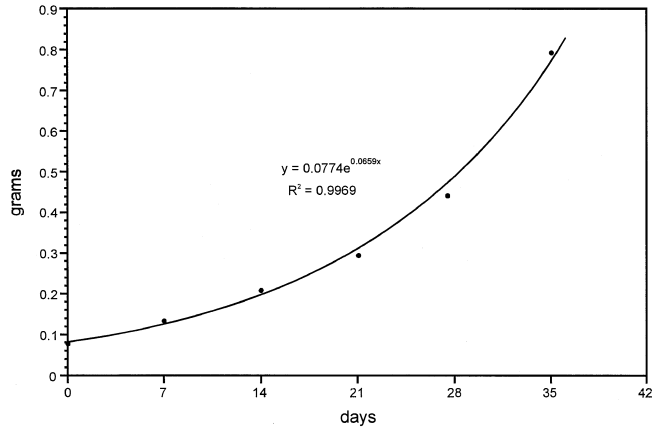

Fig. 2 Increase in fresh weight in the TIANSJ98 cell line grown on solid MPC medium. *Points* represent the mean of five samples

Chlorophyll content in the TIANSJ98 cell culture

According to Widholm (1992) the total chlorophyll content in green cultures ranges from 30 to 2,000 μg/g FW, with most cultures being below the 200 μg level. In the TIANSJ98 cell suspension and under our experimental conditions, we found that the total chlorophyll content varied between 18.3 and 121.6 μg/g FW within the growth cycle (Fig. 4). Chlorophyll content in the initial 13-day-old inoculum was 116.1 μg/g FW. Later, a decrease in chlorophyll concentration was observed during the 6 days after initiation of the experiment. Sucrose has been found to be inhibitory to chlorophyll accumulation in green cell cultures of *Spinacia oleracea* (Dalton and Street 1977), *Solanum tuberosum* (LaRosa et al. 1984) and *Euphorbia characias* (Hardy et al. 1987). Similarly, in the TIANSJ98 culture chlorophyll levels seemed to be re-established as the carbohydrate source was depleted, reaching the maximum pigment content by day 12 with 121.6, 59.0 and 62.3 μg/g FW for total, *a* and *b* chlorophyll, respectively (Fig. 4). Hüsemann (1984) considered a 300% increase in fresh weight within 10 days and a high chlorophyll content (more than 70 μg/g FW) as prerequisites for the initiation of photoautotrophic cell cultures. Although photoautototrophic growth in the TIANSJ98 line has not yet been tested, the chlorophyll levels and growth rate observed in this cell suspension are within the levels required for the initiation of photoautotrophic cultures. Contrary to the findings of other investigators (Bergmann 1967; Hüsemann and Barz 1977; Yamada et al. 1978; Chaumont and Gudin 1985), we found 2,4-D not to be detrimental to chloroplast development (Fig. 1e), at least not with the concentration of 1 mg/l that we used. However, the convenience of using other auxins (such as 1-naphthaleneacetic acid or indole butyric acid) known to be more compatible with chlorophyll accumulation remains to be tested.

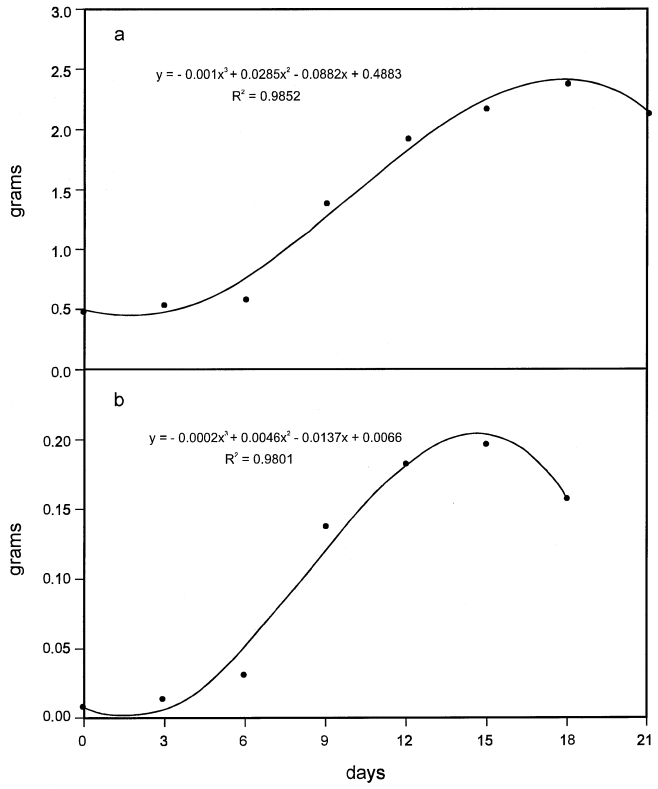

Fig. 3a,b Fresh and dry weight kinetics in the TIANSJ98 cell line grown in batch culture. **a** Fresh weight, **b** dry weight. *Points* represent the mean of six samples for each variable

Regeneration of plants

The regeneration of plants was only observed in media containing MS medium with 3% or 6% sucrose or in MS medium with 3% sucrose supplemented with 10 or 20 mg/l adenine. However, the plantlets regenerated in treatments containing adenine developed new callus at

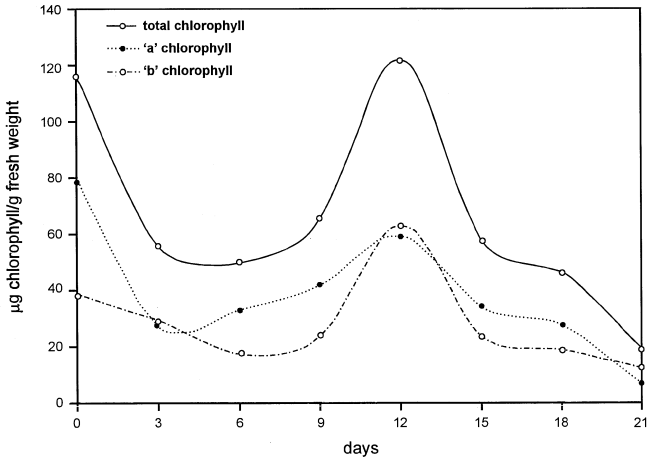

Fig. 4 Chlorophyll kinetics in the TIANSJ98 cell line grown in batch culture. *Points* represent the mean of three samples

the shoot apex region if exposed to this compound for more than 8 weeks. This regeneration took place via somatic embryogenesis (Fig. 1f–h) with the embryos showing well-developed coleoptiles (Fig. 1g). Complete plantlets were obtained 2 months after being transferred to regeneration media, but a second 2-month period was required to obtain plantlets with an adequate root system for transplantation to pots. All of the regenerated plants were established in the greenhouse and produced seed (Fig. 1i). In the best case we obtained as many as 108 embryos per 500 mg FW; however, the number of plants regenerated was significantly lower (5 plants per 500 mg FW). Experiments should be conducted to improve the response of the somatic embryos to our culture conditions. At present. we have been able to regenerate plants from the TIANSJ98 cell suspension for 1.5 years. The potential for production of regenerable green calli has been retained in plants recovered from the TIANSJ98 cell line. This green callus-forming characteristic seems to be linked to the genetic background of the TIANSJ98 cell line because in an exhaustive survey (500 shoot apices) no other chlorophyllous callus could be observed on explants obtained from different plants cultured on MPC medium. *Bouteloua gracilis* stands can be characterized by considerable intraspecific variability (McGinnies et al. 1988). Since the plant material evaluated in this study was collected in a native *B. gracilis*-dominated grassland, a great population genetic variability would explain why just one explant in 500 shoot apices responded to the culture conditions applied in this investigation. A mutation induced by the tissue culture process itself would also explain the nature of this cell line. Using regenerated plants from the TIANSJ98 cell line we observed that chlorophyllous calli can also be obtained using hormone combinations other than MPC – for example, using MS amended with 2 μM IBA, 10 or 20 mg/l adenine.

Final remarks

In an earlier study we developed the first tissue culture with regenerative capacity for blue grama (Aguado-Santacruz et al. 2000). Here we describe the production of a highly chlorophyllous and embryogenic cell culture of *B. gracilis*. Preliminary experiments in our laboratory indicate that the TIANSJ98 cell line is tolerant to osmotic stress, resisting up to 25% PEG 8000 (unpublished data). Therefore, the TIANSJ98 cell line could become an excellent model for analyzing cellular mechanisms that would enable blue grama to face water-limiting conditions in the semiarid regions of North America while studying how fundamental plant processes, such as photosynthesis, are affected by water (osmotic) stress in this very drought-tolerant plant.

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