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ORIGINAL PAPER

The effects of growth regulators and a scanning electron microscope study of somatic embryogenesis in Antartic hair grass (*Deschampsia antarctica* Desv.)

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Abstract *Deschampsia antartctica* Desv. is a type of grass that is physiologically and biochemically adapted to the extreme environmental conditions of the Antarctic continent, which is of interest to many investigators. To explore the potential use of somatic embryogenesis as a biotechnological tool for the mass micropropagation of this grass, the effects of three dosages of 2,4-dichlorophenoxyacetic acid, dicamba, and picloram were evaluated. The developmental and morphological stages of somatic embryo formation were evaluated using scanning electron microscopy (SEM). Plant regeneration was evaluated under the effects of different dosages of 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA), alone and combined. The results indicated that a Murashige and Skoog basal medium supplemented with 3 mg/l of dicamba was the best for inducing somatic embryogenesis, while the combination of 1 mg/l BAP and 0.1 mg/l of NAA was the most efficient for the regeneration and development of the plants. This work demonstrates, for the first time with the use of SEM, that it is possible to apply somatic embryogenesis for the regeneration of superficial and morphological structures of somatic embryos in the species *D. antarctica*.

Keywords *Deschampsia antarctica* Desv. · Embryogenesis · Somatic embryo · Auxin · Cytokinin · Antarctic

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Introduction

The Antarctic continent, with its extreme environmental conditions, is considered one of the most primitive ecosystems in the world. It is permanently covered by ice and snow, and only 2 % of its surface is habitable by plants (Alberdi et al. 2002). *Deschampsia antarctica* Desv. is an angiosperm that belongs to the family Poaceae and which has naturally colonized the Maritime Antarctic below 68°S, without extending to the continent, and it can also be found on the South Orkney Islands (Alberdi et al. 2002). This species is physiologically and biochemically adapted to extreme environmental conditions. Recent studies have reported certain photoprotection factors, as obtained from extracts of grass blades. These factors are possibly attributed to the actions of flavonoid and carotenoid molecules that act as absorbers of UV radiation, antioxidants, and stimulators in the processes of DNA repair (Pereira et al. 2009). These properties make this species resistant to rapidly changing environments subject to different abiotic factors such as high and low radiation, precipitation deficits, dryness, flooding, salinity, and extremely low temperatures accompanied by events of frosting, ground freezing, and layers of snow and ice (Zuñiga et al. 1996; Bravo et al. 2001; Day et al. 2001; Alberdi et al. 2002; Bravo and Griffith 2005).

The growth period begins in November, spring, and occurs through the germination of seeds or through an outbreak of tillers from previous years (Holderegger et al. 2003). Sexual reproduction is rare as flowering, and seed maturation is restricted by climatic conditions, thus limiting the completion of the gamete reproductive cycle (Ruhland and Day 2001). Given the possible relevance of *D. antarctica*, it is important to find alternatives of efficient reproduction under controlled conditions during the entire year, which would curb the issues of its naturally limited availability and its possible alterations of native habitats. The induction of embryogenetic calluses in the presence of auxins and regeneration in the presence of cytokinins, in cultivation, could be one of these alternatives. Exogenous auxins have been used in low concentrations in the growth medium to induce the formation of somatic embryos (SE) for diverse grass species, for example in *Poa pratensis* (Hu et al. 2006), *Oriza sativa* (Abe and Futsuhara 1986; Visarada et al. 2002), *Hordeum vulgare* (Chang et al. 2003; Sahrawat and Chand 2004), *Eremochloa ophiuroides* (Barampuram et al. 2009), *Zea Mays* (Huang and Wei 2004), *Lolium sp.*(Creemers-Molenaar et al. 1988), and *Saccharum sp.* (Basnayake et al. 2011), among others. In the majority of cases, 2,4-dichlorophenoxyacetic acid (2,4-D) has been the auxin used for inducing somatic embryogenesis; however, other investigators have efficiently used picloram (Sharma et al. 2007; He et al. 2010; Steinmacher et al. 2011) and dicamba (Brisibe et al. 1994; Filippov et al. 2006).

Somatic embryogenesis is a process that forms embryos from the somatic cells of plant tissue, which simulate those obtained through the gamete fusion of cells and thus conserve the capacity for developing into a plantlet when placed in the appropriate growth medium. This method has been efficient in establishing in vitro regeneration protocols for various grass species Creemers-Molenaar et al. 1988; Visarada et al. 2002; Huang and Wei 2004; Sahrawat and Chand 2004; Hu et al. 2006; (Barampuram et al. 2009; Basnayake et al. 2011); however, no structural evidence, either superficially or morphologically through scanning electron microscopy (SEM), or precedents about the use of this multiplication technique exist for the embryogenesis of this extremophile plant.

Plant reproduction through somatic embryogenesis consists of the following three stages: induction, maturation, and germination of the embryo (Kaparakis and Alderson 2008). According to Corredoira et al. (2003), in each of these stages, there are endogenous (genotype and explant type) and exogenous (composition of the growth medium, regulators of growth, and photoperiod) limiting factors. Generally, in vivo plant tissue cultivations are complemented with high concentrations of growth regulators so as to induce somatic embryogenesis (Quiroz-Figueroa et al. 2006), such as with the auxin hormone, which is considered one of the most important hormones for induction. However, the maturation and germination of the embryo normally require low concentrations or the absence of these hormones. Gaj (2004) and Raemakers et al. (1995) suggest that the most frequently used auxins for inducing SE are 2,4-D, 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), picloram, and dicamba. Other growth regulators are the cytokinins 6-benzylaminopurine (BAP), benzyladenine, 2-isopentenyladenine, zeatin, and kinetin, which do not have a clear role in the process of induction. It is believed that they are involved in the differentiation and development of morphogenetic events, therefore suggesting their importance in the stages of maturation and germination for the SE (Kaparakis and Alderson 2008).

Scanning electron microscopy is the ideal technique for high-resolution examinations of plant surfaces. Observations of SE development through stereo microscopy only offer information on changes in shape, but observations made via SEM obtain more detailed information on surface changes throughout development and permit for the perception of three-dimensional projections of the surface, tissues, and structures (Totik et al. 1998).

In this study, the effects of different concentrations of growth regulators, auxins and cytokinins, were evaluated overtime with the goal of developing viable embryos under controlled conditions in vivo. Furthermore, in order to obtain detailed evidence for the development of SE, superficial structures and morphological responses were evaluated through the use of SEM.

Materials and methods

Cultivation

Cultivation was performed in a room designated for plant microculture in the VentureLab laboratory of the Universidad Adolfo Ibáñez. The controlled conditions of the growth chamber were as follows: a temperature of 22 ± 2 °C, a white florescent light (36 W/33 Philips bulbs), and a photoperiod of 16 h with irradiation at 30 umol/m^2s^1. All of the explants were cultivated in a Murashige and Skoog (MS) (Murashige and Skoog 1962) basal medium supplemented with 3 % (w/v) sucrose (Sigma) and 2.5 g/l Gelrite (PlantMedia). The pH was adjusted to 5.7 before the addition of the gelling agent, and it was then sterilized in autoclave at 121 °C for 20 min.

Embryogenetic callus induction

The explants used in this study were obtained from *D. antarctica* tillers grown in vitro and collected from the Fildes Peninsula of King George Island in the Maritime

Antarctic (62°10′S; 58°51′W). Sterilization of the tillers was performed with 70 % ethanol (Winkler) for 20 s, 10 % sodium hypochlorite (NaClO) for 10 min, and 0.1 % of Mercury (I) chloride (Hg_2Cl) (Merk) for 5 min, with successive rinsing in sterilized, distilled water. The tillers were maintained for 1 year under controlled conditions (see cultivation method).

Subsequently, explants of 10 mm were placed in a MS solid medium in disposable 150 × 15 mm petri dishes (BD Falcon™), with 50 explants per dish, in triplicate. The medium was supplemented with concentrations of 1, 2, and 3 mg/l of the auxins 2,4-D, picloram (PhytoTechnology), and dicamba (Sigma). As a control, there was a medium without hormones. The basal meristem of each explant was exposed to each condition. The cultivation was maintained in darkness for 8 weeks, with each dish wrapped in aluminum foil. The percentage of callus formation and SE per explant was calculated at weeks 4, 6, and 8.

Regeneration and proliferation of somatic embryos

Somatic embryos obtained in the optimum condition of callus induction were isolated in a MS liquid medium using a stereomicroscope (Leica Zoom™ 2000, Leica Microsystems Inc., NY, USA). Each embryo was cultivated in a MS solid regeneration medium with concentrations of two cytokinins, alone and combined, as follows: BAP (PhytoTechnology) (0.1, 0.5, 1.0, and 3.0 mg/l), NAA (PhytoTechnology) (0.1, 0.25, 0.5, and 1.0 mg/l), and a control medium without hormones. Twenty-five embryos were seeded in disposable 94 × 16 mm petri dishes (Greiner Bio-One), and the trial was performed in triplicate. After 8 weeks of cultivation, morphogenetic responses were evaluated by calculating the number of explants that were germinated, oxidized, and with root and shoot formations. The plantlets obtained in the optimum condition of regeneration were removed from their dishes, transferred to jars with a MS solid elongation medium with 0.1 mg/l BAP and maintained for 4 weeks. Finally, the plantlets were transplanted to pots containing a mix of peat and perlite soil (3:2 v/v) and maintained in control conditions for 2 months.

Scanning electron microscopy

To observe the embryogenetic callus structures, a LEO 1420 VP scanning electron microscope (LEO Electrón Microscopía Ltd., Cambridge, UK) was used. The samples were prepared according to the methodology of Steinmacher et al. (2011) with the following modifications: embryogenetic calluses were fixed with 2.5 % (v/v) glutaraldehyde in a 0.1 M phosphate buffer (pH 7.0) for 24 h at 4 °C, with subsequent dehydration at room temperature in a series of ethanol (Merk) and acetone (Merk) concentrations (30, 50, 70, 90, and 100 % v/v in water) for further analysis. Samples were dried at the critical point in liquid CO_2 (Sorvall Critical Point Drying System, Connecticut, USA) and covered again with 99 % gold/palladium (relation Au:Pd, 60:40) (Pelco, Watford, UK) in the Sputter Pelco 91000 (Polaron Equipment Ltd., Watford, UK).

Results

After 2 weeks of cultivating *D. antarctica* in a MS medium with three concentrations of 2,4-D, dicamba, and picloram (1, 2, and 3 mg/l), the explants started to bloat in the zone that was in direct contact with the medium, zones which subsequently formed into a mass that appeared callus-like. In the following weeks of cultivation, an increase in the frequency of callus induction was observed, with greater frequency linked to higher auxin content. Between weeks 4 and 6 of cultivation, 2,4-D increased the frequency of callus induction between 68 and 84 % (1 mg/l), 77 and 87 % (2 mg/l), and 89 and 93 % (3 mg/l) (Fig. 1a). During this same time period, the effect of dicamba was slightly higher, with the frequency of induction between 75 and 95 % (1 mg/l), 79 and 90 % (2 mg/l), and 99 and 99 % (3 mg/l) (Fig. 1b). In the case of the auxin picloram, the increase was between 46 and 78 % (1 mg/l), 79 and 93 % (2 mg/l), and 80 and 89 % (3 mg/l) (Fig. 1c), which despite having a slightly lesser effect than the other hormones reached values close to 80 % or more at week 6. During week 8 of cultivation, the three hormones (2,4-D, dicamba, and picloram) obtained their greatest induction percentages (≥78 %), with the dose of 1 mg/l being 92, 99, and 78 %, respectively; the dose of 2 mg/l being 88, 93, and 97 %, respectively; and the dose of 3 mg/l being 93, 99, and 94 %, respectively (Fig. 1). On the other hand, no callus production was observed in the MS culture medium free of auxins.

Although it was possible to induce calluses with all of the evaluated auxins, not all treatments resulted in the formation of embryogenetic structures in *D. antarctica.* Only at week 8 was induction evident, and the greatest concentrations of 2,4-D and dicamba resulted in the highest percentages of induction (Fig. 2). With the use of 2,4-D and dicamba, each dose of 1, 2, and 3 mg/l resulted in an induction percentage of 8, 15, and 33 % and 3, 31, and 69 %, respectively, in the explants. The use of picloram did not result in observable structures, except for the dose of 3 mg/l which showed a 5 % induction rate in the explants. The best percentage of SE induction was only obtained by using the highest dose of dicamba (3 mg/l; 69 %).

When observing callus development through SEM in *D. antarctica* submitted to 3 mg/l of dicamba, different stages

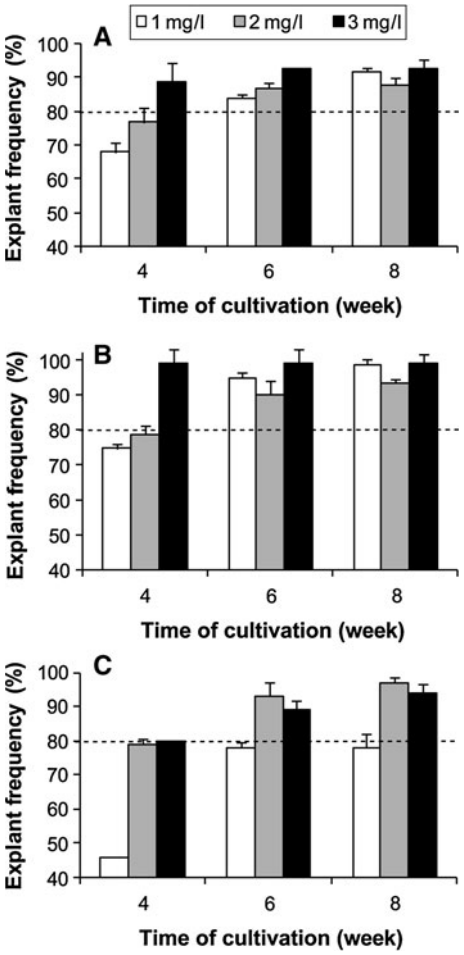

Fig. 1 Callus induction frequency in explants of *D. antarctica* Desv. in a Murashige and Skoog (MS) medium supplemented with 3 concentrations of **a** 2,4-dichlorophenoxyacetic acid (2,4-D), **b** dicamba, and **c** picloram. The 50 explants were evaluated in triplicates over the course of 4, 6, and 8 weeks after the start of cultivation. Frequency values are expressed as the percentage of explants that showed callus formation. During week 8 of cultivation, the three hormones obtained their greatest induction percentages (≥78 %). Results are presented as a mean of induction percentage ($n = 3$) ± SD

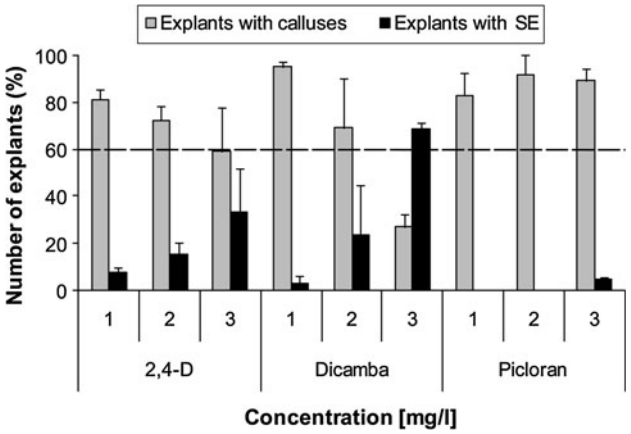

Fig. 2 Number of *D. antarctica* explants with callus formation in a MS medium supplemented with 3 concentrations of auxins (2,4-D, dicamba, and picloram). Frequency values are expressed as a percentage of explants that presented callus formation. *Grey* explants with calluses, and *Black* explants with somatic embryos (SE). The 50 treated explants were evaluated in triplicates after 8 weeks of cultivation. The greatest percentage of SE induction was obtained by using a 3 mg/l dosage of dicamba (69 % ± 3). Results are presented as a mean of SE induction ($n = 3$) ± SD

of somatic embryogenesis could be distinguished. During the first 4 weeks of cultivation, callus formation with no defined structures was observed (Fig. 3a); however, after 6 weeks of cultivation, the initial formation of globular structures in different zones of the callus was evident (Fig. 3b, c), resulting in the development of somatic embryo clusters (Fig. 3d). During the maturation of the SE, complete globular structures with round cells were observed (Fig. 3e, f).

Figure 4 shows the regeneration sequence of the SE of *D. antarctica*, including the initial explants (Fig. 4a) and the embryogenetic calluses induced with dicamba (3 mg/l), which was the most effective auxin in stimulating SE (Fig. 4b). Clusters with well-defined globular structures (Fig. 4c) were dispersed in a MS liquid medium (Fig. 4d), liberating cells in suspension, which were possibly superficial cells that helped to maintain the cluster form of the SE. These structures were cultivated in a MS solid medium with different combinations of BAP (0.1, 0.5, 1.0, and 3.0 mg/l) and NAA (0.1, 0.25, 0.5, and 1.0 mg/l).

In all treatments, the addition of only BAP in dosages between 0.5 and 3 mg/l promoted germination up to 49 ± 6 % in the SE, and when combined with dosages between 0.1 and 0.5 mg/l of NAA, this increased up to 60 ± 4 % (Fig. 5a). On the other hand, dosages of NAA higher than 0.5 mg/l provoked oxidation in the SE, with between 39 ± 6 % for 0.5 mg/l and 53 ± 9 % for 1.0 mg/l, and when combined with concentrations of BAP higher than 1.0 mg/l, oxidation reached 40 ± 7 % (Fig. 5b).

At 20 days of cultivation, the presence of the first roots was observed (Fig. 4e) for 12 ± 4 % of the SE that underwent treatments with only NAA and in combination treatments with dosage levels of BAP. On the other hand, no roots were observed with treatments of only BAP (Fig. 5c). The first shoots were observed during week 4 of cultivation in the regeneration medium (Fig. 4f), and at week 8, completely formed plantlets were observed (Fig. 4g). The morphogenetic response, expressed by the number of shoots developed from germinated embryos, was greater in the treatments supplemented with 1.0 and 3.0 mg/l of BAP (between 40 ± 4 % and 44 ± 7 %, respectively) (Fig. 5d). When combining this with a dose of NAA, the percentage of shoots diminished in correlation with an increase in the dosage levels of auxin, arriving at

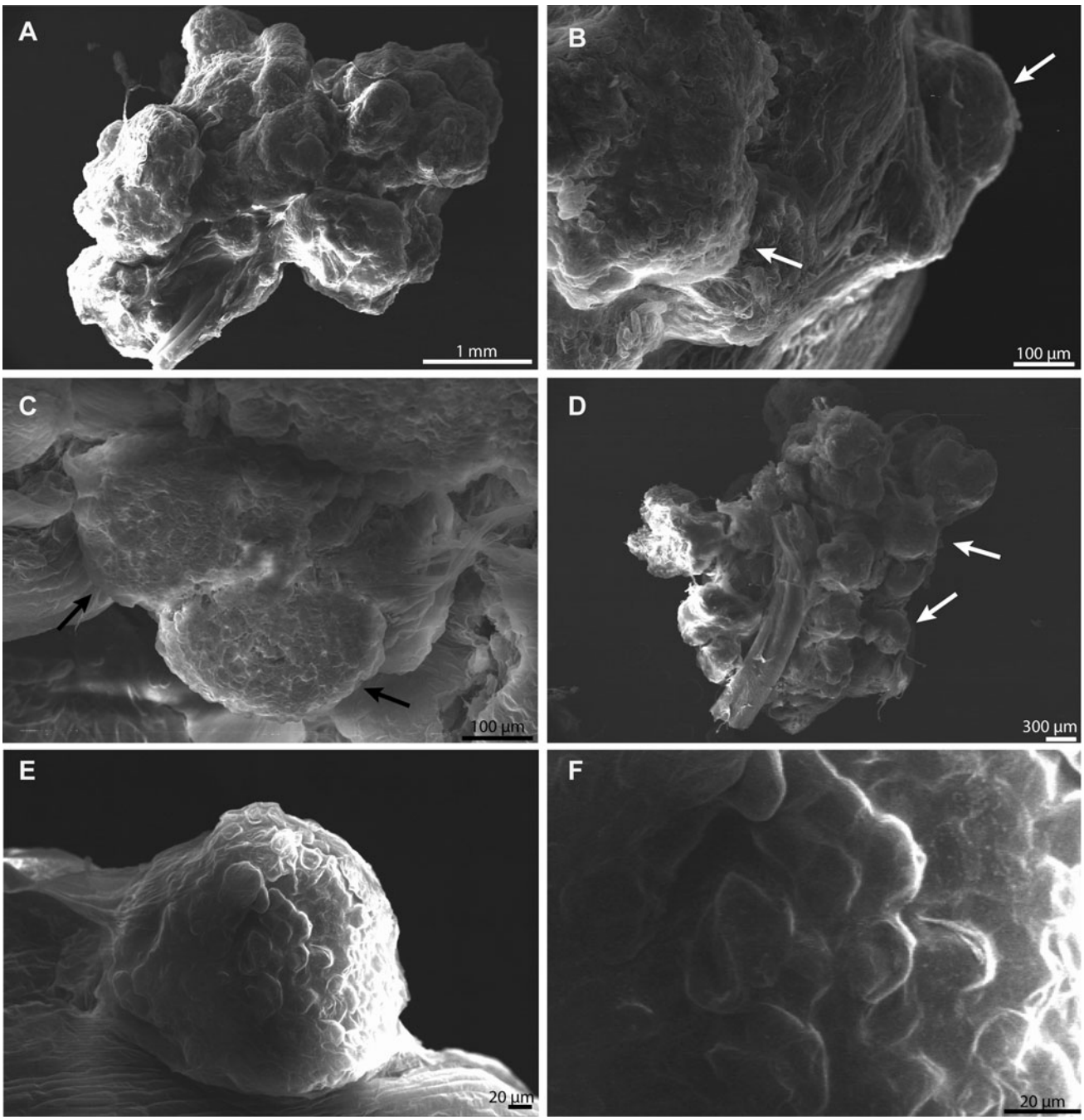

Fig. 3 Scanning electron microscopy (SEM) detailing the formation of SE in *D. antarctica*. **a** Initial development of undefined callus, **b** callus with initial globular formation, **c** groups of SE in development, **d** callus with globular and more developed SE, **e** developed somatic embryo with a complete globular structure, and **f** superficial detail of the globular somatic embryo, where observed cells have a *rounded shape*

12 ± 4 %. The number of shoots was null or very low (4 %) in all cases where the medium was only complemented with NAA.

When combining the results concerning quantity of germinated and oxidized SE, formation of roots, and number of shoots per embryo (Fig. 4), it was observed that the combination of 1 mg/l BAP and 0.1 mg/l NAA permitted the greatest percentage of germination (60 ± 4 %), a low oxidation (10 ± 3 %), and some of the greatest percentages root development (27 ± 8 %) and shoots per embryo (40 ± 4 %) in *D. antarctica*.

Afterwards, plants obtained from the SE of *D. antarctica* were cultivated in a MS solid medium with 0.1 mg/l BAP to promote elongation (Fig. 4h), and they were finally

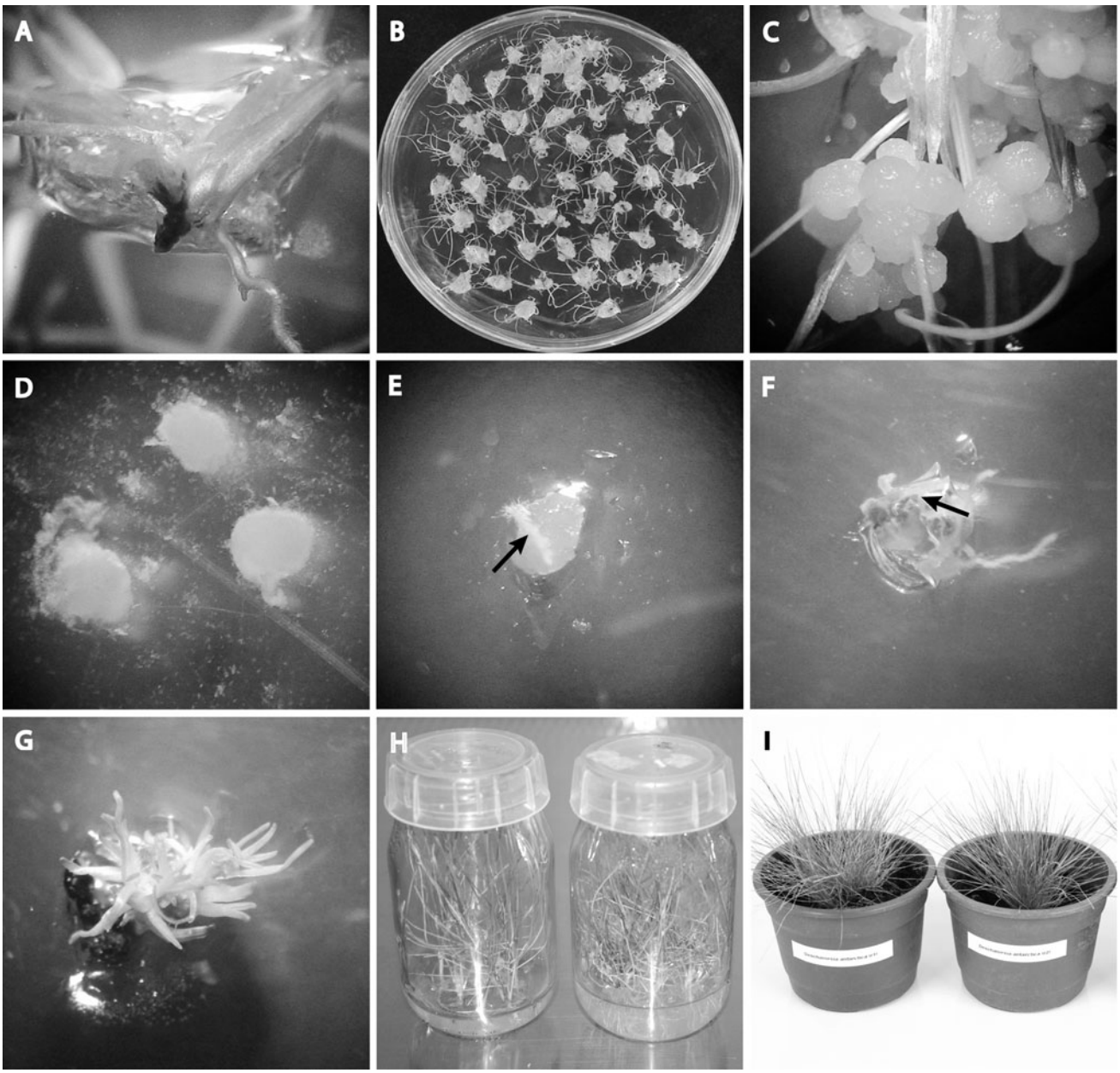

Fig. 4 Image sequence showing the regeneration of *D. antarctica* generated from SE. **a** Inferior view of the explant detailing the meristem region, **b** inferior view of the cultivation dish with explants with developed calluses (dicamba, 3 mg/l, week 8), **c** differentiated structures forming clustered SE (dicamba, 3 mg/l, week 8), **d** detail of SE separated from the bunch and floating in a MS liquid medium, the debris indicates tissue uniting the embryos, **e** embryo placed in a MS *solid medium*, the *arrow* indicates the rootlet obtained after 3 weeks, **f** embryo at 4 weeks, the *arrow* indicates new shoots, **g** completely formed plantlet after 6 weeks, **h** plantlet in a MS *solid medium* supplemented with 0.1 mg/l BAP, the *arrow* indicates elongated leaves, **i** plantlets generated from SE placed in soil

moved to a plant pot with soil where they showed phenotypic characteristics similar to the mother plant (Fig. 4i).

Discussion

When cultivating *D. antarctica* in three concentrations of 2,4-D, dicamba, and picloram, there was an evidently high callus induction frequency (≥78 %) during a period of 8 weeks; however, the dicamba dosage of 3 mg/l was observed to be the most effective for the induction of somatic embryos in this species. A similar case was reported in *H. vulgare* (Chang et al. 2003). For cultivations of some grass species, dicamba has been seen as superior to the auxin 2,4-D for inducing SE (Trifonova et al. 2001; Przetakiewicz et al. 2003). *D. antarctica* can probably

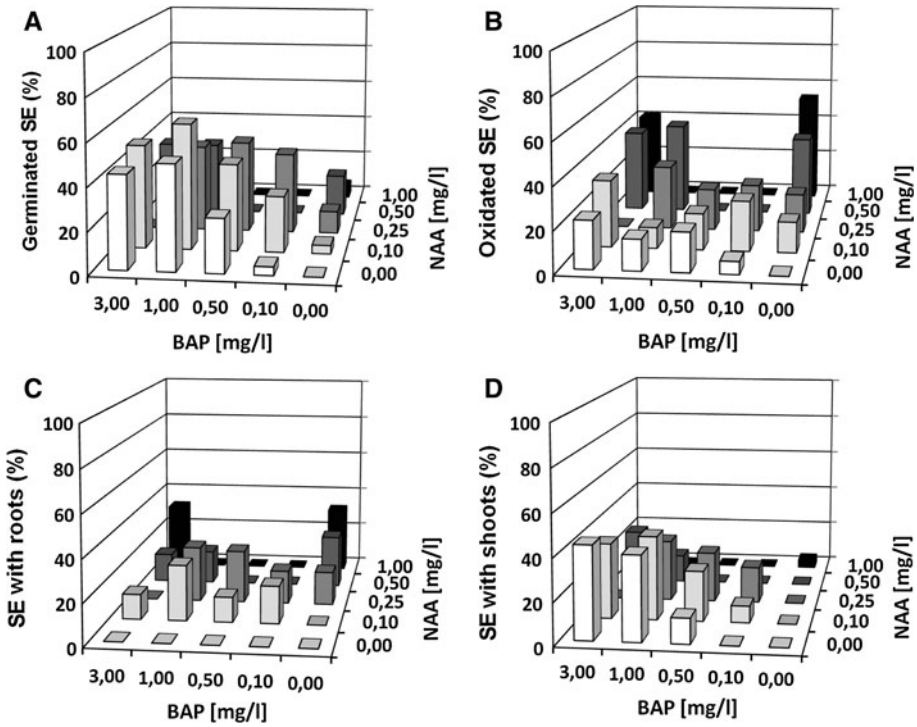

Fig. 5 Image representing the effects of the combined concentrations of the cytokinin BAP and auxin NAA on the proliferation and regeneration of plantlets generated from the somatic embryos of *D. antarctica*. The *bars* represent the percentage of embryos according to: **a** germinated SE, **b** oxidized SE, **c** SE with roots, and **d** SE with shoots. The combination of 1 mg/l BAP and 0.1 mg/l NAA permitted the greatest percentage of germination, low oxidation, and some of the greatest percentages for obtained roots and shoots per embryo

metabolize the auxin dicamba faster than 2,4-D or picloram, thus permitting the stimulation of greater promoter activity in mitotic divisions. During the transition to somatic embryogenetic, plant cells have to differentiate, activate their cellular division cycle, and reorganize their physiology, metabolism, and patterns of gene expression (Fehér et al. 2003). As a consequence of this, the cells capable of producing SE are mitotically more active than non-embyrogenetic cells (Pasternak et al. 2002).

Some studies reveal that exogenous auxins are necessary for the first stage of embryogenesis (formation of pro-embryogenetic masses); however, following stages (transition of callus to somatic embryo) require a decrease or deprivation of these in the growth medium (Choi et al. 1998). This gives reason to think that explants of *D. antarctica* fully use the more easily metabolized auxin dicamba, thus permitting the precursory embryo masses to continue with their development until forming SE (maturation).

The morphology of an embryogenetic callus varies according to the species. Via scanning electron microscopy, the details of precursory embryo masses in different phases of SE induction showed the development of isolated globular structures from initiation until maturation. These masses clearly resulted in small groups of globular structures shaped by round cells typical of embryogenetic tissue. Similar observations were obtained in other grasses where paths of reproduction were studied in vitro and then examined by SEM (Rodriguez et al. 1996; Li and Qu 2002; Steinmacher et al. 2011).

As expected, when cultivating the obtained globular structures SE of *D. antarctica*, almost all of the used BAP dosages resulted in the germination of shoots, and when combined with NAA, the formation of roots was observed in all cases. On the other hand, NAA by itself promoted the formation of roots only in high concentrations, but it increased the rate of oxidation in the embryos. In addition, high concentrations of BAP also had an evident oxidative effect, despite a high percentage of shoot formation. It is possible that under these conditions, both hormones have a phytotoxic effect on the SE of this grass. However, discrete doses (1.0 mg/l of BAP and 0.1 mg/l of NAA) permit a high germination rate (60 %) with minimum oxidation (10 %). In some grass species cultures, such as *H. vulgare* (Chang et al. 2003), *P. pratensis* (Hu et al. 2006), and *E. ophiuroides* (Liu et al. 2008), the influence of these growth regulators in the regeneration of SE has been studied. While the results of these studies are varied, some results are similar results to those currently presented. The administration of exogenous cytokinins in combination with low concentrations of auxins has an essential role for growth and post-embryogenetic development (Muller and Sheen 2008).

Some studies have described the antagonistic effect of cytokinins and auxins in the establishment of the root stem-cell niche of the model plant *Arabidopsis thaliana* (Muller and Sheen 2008; Perilli et al. 2010). It is possible that this same effect occurs in *D. antarctica* with the discrete combination of evaluated hormones. BAP could be

controlling the rate of cellular differentiation during the development of the root meristem when signaling transportation, such as of NAA, is so suppressed, whereas in the early stages of embryo development the auxin impedes the signaling of BAP so as to establish an embryonic root stem-cell niche. Deeper studies of this interaction, for example of genetic expression, could corroborate hormonal behavior.

Finally, all of the plantlets obtained in this study positively responded to transplantation into soil, before the elongation stage. Although all individuals demonstrated phenotype characteristics similar to the mother plant, greater levels of germination could be obtained by bettering the conditions of embryo maturation.

In conclusion, dicamba optimally induced the development of viable somatic embryos, and the discrete combination of BAP and NAA permitted a high percentage of regeneration. Due to this, somatic embryogenesis is a feasible technique for the micropropagation of *D. antarctica* under controlled conditions. The use of SEM demonstrated for the first time superficial and morphological structures of somatic embryos from this species. This work establishes micropropagation conditions under which *D. antarctica* can be cultivated to provide plant matter for future investigations and for biotechnological applications with artificial seeds, transgenic plants, etc., without altering the natural habitat.

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