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Fertile plant regeneration from protoplasts of meadow fescue (*Festuca pratensis* Huds.)

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ABSTRACT

Suspension cultures from mature embryo-derived compact callus were initiated in seven meadow fescue (*Festuca pratensis* Huds.) cultivars. Four to six months after initiation, embryogenic suspension cultures with a moderate growth rate were established from three of them (cvs. Barmondo, Belimo and Leopard). These suspension cultures showed the capacity, maintained over six months, to regenerate green plants which could be grown to maturity under greenhouse conditions.
Morphogenic suspension cultures from single genotypes of three *F. pratensis* cultivars (cvs. Barmondo, Belimo and Leopard) yielded large numbers of protoplasts, which upon culture in agarose beads using nurse cells formed microcalli with an overall plating efficiency in the range of 10^{-3} to 10^{-4}. Mature plants were reproducibly regenerated and established in soil, from such protoplasts during a period of six months.
The regeneration of fertile plants from protoplasts derived from suspension cultures of meadow fescue and its implications on gene transfer technology for this species are discussed.

Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid.

Key words: forage grasses - *Festuca pratensis* - suspension cultures - protoplasts - plant regeneration

INTRODUCTION

Meadow fescue (*Festuca pratensis* Huds.) is a major cool-season forage grass of agricultural importance in the temperate zone. It has a wide distribution range in the northern hemisphere, mainly in Europe (Hulten 1971). Since meadow fescue generally has good winter hardiness and longevity under a system with frequent cutting or grazing, it has become an increasingly important crop species in leys and pastures (Aastveit and Aastveit 1989).
Cereal crops and forage grasses are among the most difficult species to genetically manipulate *in vitro* (Vasil 1988; Potrykus 1990). The establishment of morphogenic cell suspensions has proven to be an important prerequisite for gene transfer into these monocotyledonous species (Vasil 1988; Potrykus 1991). These cell suspensions are required for the isolation of totipotent protoplasts, which can be subjected to direct gene transfer and fusion, for the production of transgenic plants and somatic hybrids, respectively. In addition, suspension cultures can be used as targets for biolistic transformation finally leading to regeneration of transgenic plants.
Regeneration of mature, greenhouse-grown plants from protoplasts of forage grasses is so far restricted to a few species: *Festuca arundinacea* (Dalton 1988a, b; Takamizo et al. 1990), *Lolium multiflorum* (Dalton 1988b), *Lolium perenne* (Dalton 1988a, b; Creemers-Molenaar et al. 1989), *Dactylis glomerata* (Horn et al. 1988), *Agrostis alba* (Asano and Sugiura 1990), *Agrostis palustris* (Terakawa et al. 1992) and *Paspalum dilatatum* (Akashi and Adachi 1992). Plant regeneration from suspension cultures and protoplasts from meadow fescue has not been described.
In addition, no report on the fertility of protoplast-derived plants for any forage grass species has yet been published. This is, however, a key prerequisite for the generation of materials

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proposed to have an agricultural impact and thus requiring their inclusion in further breeding, propagation and commercial seed production programs.
Here we describe the reproducible regeneration of mature plants from suspension cultures and fully fertile plants from protoplasts of *Festuca pratensis*.

MATERIALS AND METHODS

Plant material

Seeds from 10 meadow fescue (*Festuca pratensis* Huds.) cultivars Barkas, Barmondo, Barpresto, Bartran, Belimo, Cosmos II, Leopard, Predix, Prefest and Stella were provided by J. Lehmann and B. Boller, FAP Zürich-Reckenholz, Switzerland and used for production of embryogenic callus. Flowering plants from meadow fescue ecotype "Les Mottes" were made available by J. Nösberger, ETH-Zürich, Switzerland.

Establishment and maintenance of suspension cultures

After surface sterilization in 3% (w/v) calcium hypochlorite for 40 min, seeds were rinsed in sterile water (3x) and placed on Murashige and Skoog (1962) medium (MS medium) supplemented with 500 mg/l casein hydrolysate, 5 mg/l 2,4-D, 3% (w/v) sucrose, solidified with 1% (w/v) agar (agar-agar Merck for microbiology) and were kept in the dark at 25°C. Callus appearing within 5-8 weeks after inoculation of seeds was maintained by subculturing on the same medium every 4-6 weeks. After one subculture, friable, yellowish, embryogenic callus derived from single seeds (representing individual genotypes) was individually transferred to 6-well culture dishes (Falcon 3046, Becton Dickinson & Co., New Jersey, USA) with 3 ml liquid AAF medium/well: AA medium (Müller and Grafe 1978) containing 1.5 mg/l 2,4-D, 2% sucrose and 3% sorbitol. Alternatively, embryogenic suspension cultures were initiated in MS medium containing 3% sucrose and 1.5 mg/l 2,4-D.
The suspension cultures initiated from individual seeds were kept at 25°C in the dark on a gyratory shaker at 60 rpm, and subcultured weekly by replacing 2/3 of the culture medium with fresh one. After four weeks, the suspension cultures initiated from individual genotypes were separately transferred to 6 cm culture dishes (Greiner 967161, 190 ml, Greiner GmbH, Nürtingen, FRG), kept under the same culture conditions and thereafter subcultured every 7-10 days.

Plant regeneration from suspension cultures

Embryogenic cell suspensions were plated on solid MS medium supplemented with 2 mg/l 2,4-D and 3% sucrose and proliferated for 3-4 weeks. Regeneration of plantlets from somatic embryos formed on the surface of compact callus was achieved after transferring the cultures onto solid MSY medium: hormone-free MS medium containing 1 g/l yeast extract, supplemented with 3% sucrose. The regenerated shoots, with or without roots, were again transferred to MS hormone-free medium in order to stimulate rooting. All cultures were kept at 25°C under a photoperiod of 16 h fluorescent light (40 $\mu E\ m^{-2}\ s^{-1}$). After 3-4 weeks, rooted plantlets were hardened off, transferred to soil and grown to maturity under greenhouse conditions (16/8 h photoperiod; 23°C/18°C; fluorescent light, 145 $\mu E\ m^{-2}\ s^{-1}$).

Protoplast isolation, culture and plant regeneration

Isolation of protoplasts was performed from established cell suspensions, 5-12 months after initiation. Protoplasts were isolated by resuspending 2-4 ml settled volume of suspension culture derived from single genotypes, 4 days after subculture, in 12-14 ml of CPW solution (Frearson et al. 1973) containing 2% (w/v) cellulase Onozuka RS; 1% (w/v) Macerozyme; 0.1% (w/v) Pectolyase Y-23 and 0.6 M mannitol. After 4 h incubation in the dark at 25°C on a gyratory shaker at 30 rpm, the incubation mixture was filtered first through 100 µm- and later through 50 µm-sieves and the protoplasts were washed twice by centrifugation at 100g for 5 min in CPW solution containing 0.6 M mannitol. Protoplasts were cultured in AA medium (Müller and Grafe 1978) containing 1 mg/l 2,4-D and 0.6 M glucose using agarose bead type culture (Shillito et al. 1983) with nurse cells, as previously described (Kyozuka et al. 1987). 1.5 ml of the suspension of protoplasts (1-2 x 10^6/ml) in two-fold concentrated protoplast culture medium containing 0.6 M glucose were mixed with an equal volume of molten 2.4% Sea-Plaque agarose (FMC Corp., Rockland ME, USA) in 0.6 M glucose and plated in a 3 cm culture dish (Falcon 3001, Becton Dickinson & Co., New Jersey, USA). The solidified agarose medium was transferred into a 6 cm culture dish (Greiner, 190 ml) containing 12 ml protoplast culture medium supplemented with 1 mg/l 2,4-D and 0.6 M glucose. About 300-400 mg of non-morphogenic suspension-culture cells of *F. pratensis* cv. Trader were used as nurse cells during the first week in culture and then removed by washing the agarose beads three times with fresh protoplast culture medium. Cultures were kept on a gyratory shaker at 50 rpm under continuous dim fluorescent light (20 $\mu E\ m^{-2}\ s^{-1}$).
After 4-5 weeks of culture, agarose beads containing visible colonies or individual colonies growing on the agarose beads were transferred onto MS proliferation medium with 2 mg/l 2,4-D and 3% sucrose. After a further 3-4 weeks, calli were placed on MS regeneration medium (MSY) without yeast extract but supplemented with 0.2 mg/l kinetin. Regeneration of mature plants from protoplasts was as indicated above for suspension cultures. Regenerated plants were vernalized at 6°C for 8 weeks. Pollen viability was determined by assessing the percentage of dark-blue stained pollen grains after staining with lugol solution (Fluka).

RESULTS AND DISCUSSION

Suspension cultures

Seven cultivars out of the 10 tested, produced embryogenic calli (Fig. 1). The establishment of

compact callus suitable for the initiation of embryogenic cell suspensions was not possible for three meadow fescue cultivars (cvs. Barkas, Barpresto and Predix), after a preliminary screening involving 20 to 300 different genotypes for each of them. Since meadow fescue is a widely self-sterile out-crossing perennial and thus expected to be highly heterozygous, individual seeds from the same cultivar represent different genotypes.

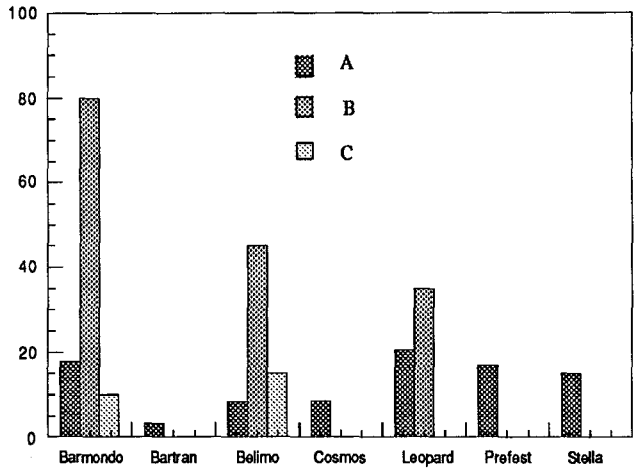

Figure 1: Frequency of embryogenic callus formation and plant regeneration from suspension cultures of different cultivars of *Festuca pratensis*. A) Number of embryogenic calli/number of plated seeds (%); B) Number of calli regenerating green plantlets/number of plated calli from suspension cultures (%); C) Number of calli regenerating albino plantlets/number of plated calli from suspension cultures (%).

Depending on the cultivar, 3 to 20% of the seeds tested for the other 7 cultivars, gave sectors of friable yellowish callus, which were used for the initiation of embryogenic suspension cultures. Suspension cultures were initiated from compact callus derived from mature embryos of defined single-seed origin from the seven *F. pratensis* cultivars. These cultures varied considerably in their growth response and degree of dispersion. Regenerable cell suspensions (16-24 weeks old) of moderate growth rate, consisting mainly of compact medium sized (< 2 mm) proembryogenic cell clusters (Fig. 2A),were established for five different genotypes from three meadow fescue cultivars (cvs. Barmondo, Belimo and Leopard) using suspension culture medium AAF. More compact cell clusters were obtained when MS based medium was used for the establishment and maintenance of suspension cultures of the same three cultivars. The observed differences in response while initiating embryogenic suspension cultures for particular cultivars of this species could be due to genotype-effects. Consequently, a further screen for responsive genotypes based on independent seed-derived calli could allow the establishment of embryogenic suspension cultures for specific meadow fescue cultivars of particular agronomic relevance and thus reduce the cultivar-dependent limitations observed. Similar genotype-dependent differential responses in the establishment of embryogenic suspension cultures have been described for other forage grasses, namely *Festuca arundinacea* (Takamizo et al. 1990) and *Agrostis alba* (Asano and Sugiura 1990).

Protoplast culture

Protoplasts were readily isolated from 5 to 12 months old morphogenic cell suspensions initiated from independent genotypes of three meadow fescue cultivars, cvs. "Barmondo", "Belimo" and "Leopard" (Fig. 2AB). Protoplasts were routinely obtained with a yield of 0.5-1 x 10^6 protoplasts/g fresh weight cells, when AAF medium was used for growth of donor cell suspensions (Fig. 2B). However, only few protoplasts could be isolated from suspension cultures consisting of more compact cell clusters when MS based medium was used for establishment and maintenance of cell suspensions.
The overall plating efficiency (number of visible colonies/number of plated protoplasts) in bead type culture was in the range of 10^{-3} to 10^{-4} provided nurse cells were used during the first week in culture. Colonies growing in the agarose beads were visible after three weeks of culture and generated a lawn on the agarose-solidified medium after 4 to 5 weeks (Fig. 2CD).

Plant regeneration and fertility

Suspension cultures obtained from responsive genotypes and maintained in AAF medium for over 6 months retained their potential for regeneration of green plantlets upon their transfer onto hormone free MSY medium. The frequency of albinism varied from cultivar to cultivar, being between 10 to 20% for suspension cultures of similar age (20 weeks old) established from cvs. "Barmondo" and "Belimo", while only green plantlets were recovered from cv. "Leopard" (Fig. 1). Thus, the frequency of albinism among regenerated *in vitro* plantlets from embryogenic suspension cultures of *F. pratensis* was within the range of previous reports for other forage grass

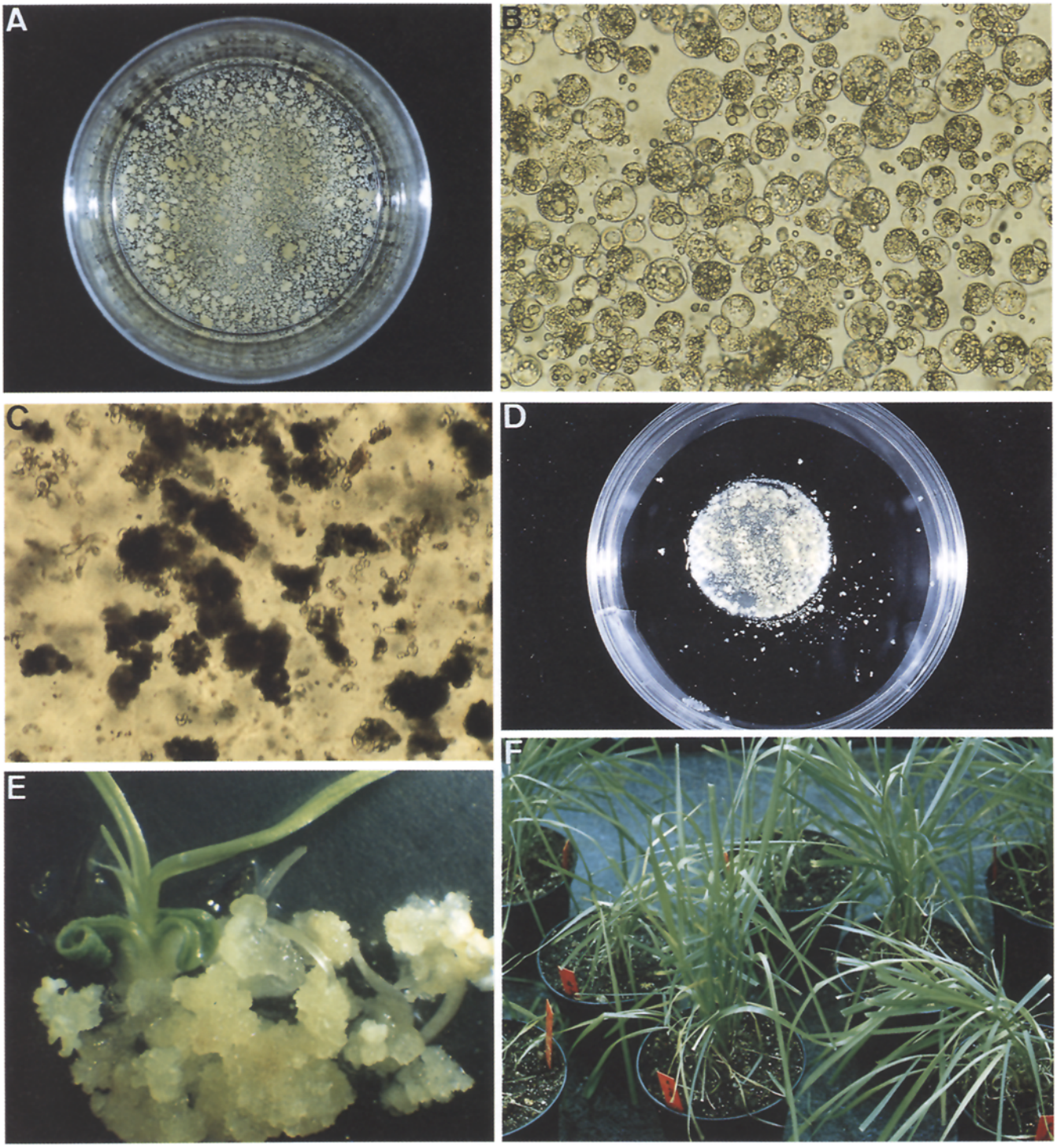

Figure 2: Plant regeneration from protoplasts isolated from embryogenic cell suspensions of *Festuca pratensis* cv. Barmondo.
A) Embryogenic cell suspension culture used for protoplast isolation five months after initiation of cultures; B) Freshly isolated protoplasts from morphogenic cell suspensions; C) Protoplast derived microcolonies 2 weeks after embedding in agarose-solidified medium; D) Bead type culture of protoplasts forming visible colonies 4 weeks after embedding; E) *In vitro* plantlet regeneration from protoplast-derived callus 3 months after protoplast isolation; F) Vernalized plants of meadow fescue regenerated from protoplasts, growing in the greenhouse.

species, being less than 5% for *Agrostis alba*(Asano and Sugiura 1990); between 5% and 20% for *Festuca arundinacea* (Dalton 1988a) and up to 80% for *Lolium perenne* (Dalton 1988a; Creemers-Molenaar et al. 1989), depending on the genotype and age of the cell suspension used.

The cryopreservation of embryogenic cell suspensions from single genotypes could further extend their availability for regeneration of green plants and as a source of totipotent protoplasts. This has been successfully demonstrated for maize (Shillito et al. 1989). Experiments on this line are now in progress with meadow fescue.
Plant regeneration from protoplast derived calli of some specific genotypes from the cultivars "Barmondo" and "Belimo" was possible, with frequencies in the range of 20 to 30% (number of green plantlets/number of colonies plated on regeneration medium) (Fig. 2EF). Some albino plantlets were regenerated from protoplasts and their frequency varied between 10% and 25% depending on the age of the cell suspension used. Thus, a similar behavior and cultivar-dependence is apparent when comparing plant regeneration directly from embryogenic suspension cultures with regeneration from the corresponding protoplasts. Therefore, in the context of gene transfer technology and the necessary tissue culture basis, no significant difference in cultivar-dependence with respect to plant regeneration capacity seems to be expected between particle bombardment of embryogenic suspension cultures and direct gene transfer to protoplasts for this species.

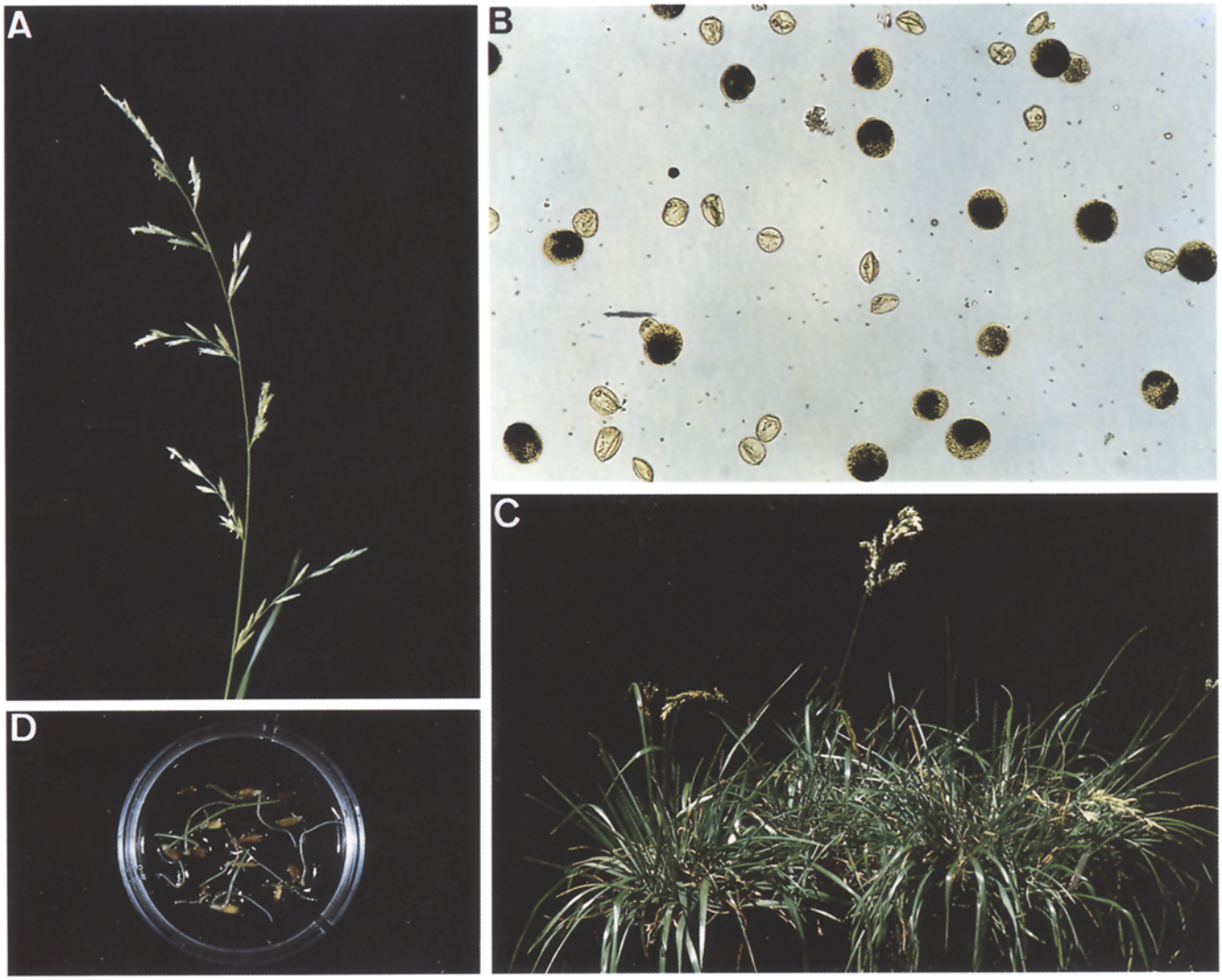

Figure 3: Fertility of plants regenerated from protoplasts of *Festuca pratensis* cv. Barmondo.
A) Inflorescence of protoplast-derived plant of meadow fescue with protruding anthers; B) Fertile pollen stained with lugol-solution from a protoplast-derived plant of meadow fescue; C) Inflorescences setting seeds on plants of meadow fescue recovered from protoplasts; D) Germinating seeds obtained from outcrossing protoplast-derived plants.

Since meadow fescue requires cold-treatment for flowering, seven plants of cv. Barmondo regenerated from protoplasts were vernalized in order to assess their fertility. The first two flowering protoplast-derived plants so far evaluated from this set, developed normal inflorescences with protruding anthers (Fig. 3A). Anthers containing up to 40% viable pollen dehisced and thus illustrate the potential for regeneration of male fertile protoplast-derived plants in *F. pratensis* (Fig. 3B). Since meadow fescue is a highly self-sterile allogamous species, flowering protoplast-derived plants were crossed with alien pollen to assess their female fertility and seed setting (Fig. 3C). In addition pollen from protoplast-derived plants was used for pollination of seed grown meadow fescue plants from ecotype "Les Mottes". Crosses in both directions succeeded and led to production of mature seeds which could be germinated (Fig. 3D).

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