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BRIEF COMMUNICATION

Somatic embryogenesis or shoot formation following high 2,4-D pulse-treatment of mature embryos of *Paspalum scrobiculatum*

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Abstract

Mature zygotic embryos of *Paspalum scrobiculatum* L. cv. PSC 1 on MS or N_6 nutrient medium supplemented with various concentrations of 2,4-D (4.5 - 22.5 μM) formed embryogenic callus, which differentiated into somatic embryos within 5 weeks of culture. The somatic embryos after transfer to hormone-free regeneration medium germinated and formed plantlets. Of the two nutrient formulations, N_6 was relatively better than MS for somatic embryogenesis. A culture for 11 d on 100 μM 2,4-D was essential for the establishment of an embryogenic callus. Shorter duration, 4-d or 7-d culture on 2,4-D medium, supported some proliferation and subsequent differentiation into shoot-buds or multiple-shoots, in high-frequency cultures. This is first instance in monocots of a controlled regeneration response; either somatic embryogenesis or shoot formation.

Additional key words: auxin, graminaceous plants, millet, organogenesis, regeneration.

Callus cultures capable of somatic embryogenesis are possible in graminaceous plants when the explants employed are "embryonic" in nature, such as unemerged inflorescences, immature zygotic embryos or in some instances the leaf bases and mature embryos. Employing such explants somatic embryogenesis has been possible in *Triticum aestivum*, *Zea mays*, *Secale cereale*, *Pennisetum americanum*, and *Panicum miliaceum* (for review, see Vasil 1985, Bhaskaran and Smith 1990). Besides an embryonic explant, another prerequisite for the initiation of an embryogenic callus culture is relatively high concentration of a synthetic auxin such as 2,4-D. This is true for rice (Abdullah *et al.* 1986), wheat (Vasil *et al.* 1990), maize (Morocz *et al.* 1990, Garcia and Molina 2001), and barley (Havrlentová *et al.* 2001). These studies emphasize that an auxin treatment is necessary for the cells to embark upon somatic embryogenesis. However, the main question that remains to be addressed is, whether cells are preprogrammed for embryogenesis or are diverted towards embryogenesis when they encounter auxin in the medium.

Previous reports on *Paspalum scrobiculatum* describe the induction of embryogenic callus from immature inflorescence explants (Nayak and Sen 1989, Vikrant and Rashid 2001). Also there is direct differentiation of somatic embryos from inflorescence axis (Vikrant and Rashid 2001). Shoot-apex explants of this millet crop are reported to be highly regenerative in long term cultures (Arockiasamy *et al.* 2001). In this study on *Paspalum scrobiculatum* mature zygotic embryos were employed as explants; these are available more easily than immature inflorescences. The questions addressed are: *1*) is somatic embryogenesis all-or-none response and *2*) is a cell/tissue preprogrammed for embryogenesis. An analysis of the requirement of exogenous auxin for somatic embryogenesis has revealed that 2,4-D has dual role in regeneration. A transient treatment of explant with high auxin resulted in multiple shoots whereas long term

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Abbreviations: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; MS medium - medium of Murashige and Skoog (1962); N6 medium - medium according to Chu *et al.* (1975).

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treatment resulted in formation of embryogenic callus and subsequent differentiation of somatic embryos. This study forms the first instance in monocots of a controlled regeneration response; somatic embryogenesis or organogenesis.

Seeds of *Paspalum scrobiculatum* L. cv. PSC 1, an important millet crop, procured from University of Agricultural Sciences, Bangalore India, were scarified with H_2SO_4 (30 %, v/v) for 30 min, followed by sterilization with $HgCl_2$ (0.1 %, m/v) for 5 min. These seeds were thoroughly washed with sterile distilled water (SDW) and finally soaked in SDW for 48 - 72 h. The partially swollen mature embryos which emerged from the seed coat (Fig. 1*A*) were excised and cultured aseptically on nutrient media.

The basal medium of Murashige and Skoog (1962; MS) or of Chu *et al.* (1975; N_6) was supplemented with 2,4-D (4.5, 9.0, 18.0 and 22.5 μM) and sucrose (2 %, m/v). This medium was gelled with *Difco* bacto-agar (0.8 %, m/v) and after adjusting the pH to 5.8 and it was sterilized by autoclaving at 120 °C for 15 min. For each treatment at least 50 replicate cultures were raised. The values given are means of four independent experiments. The cultures were incubated under continuous illumination from fluorescent tubes (irradiance of 15 μmol m^{-2} s^{-1}) at temperature 25 ± 2 °C.

In order to study the pulse-effect of high 2,4-D concentration on morphogenesis, mature embryos were cultured on N6 medium supplemented with 100 μM 2,4-D for different durations (2, 4, 7, 11 and 21 d) and then transferred to N_6 basal medium.

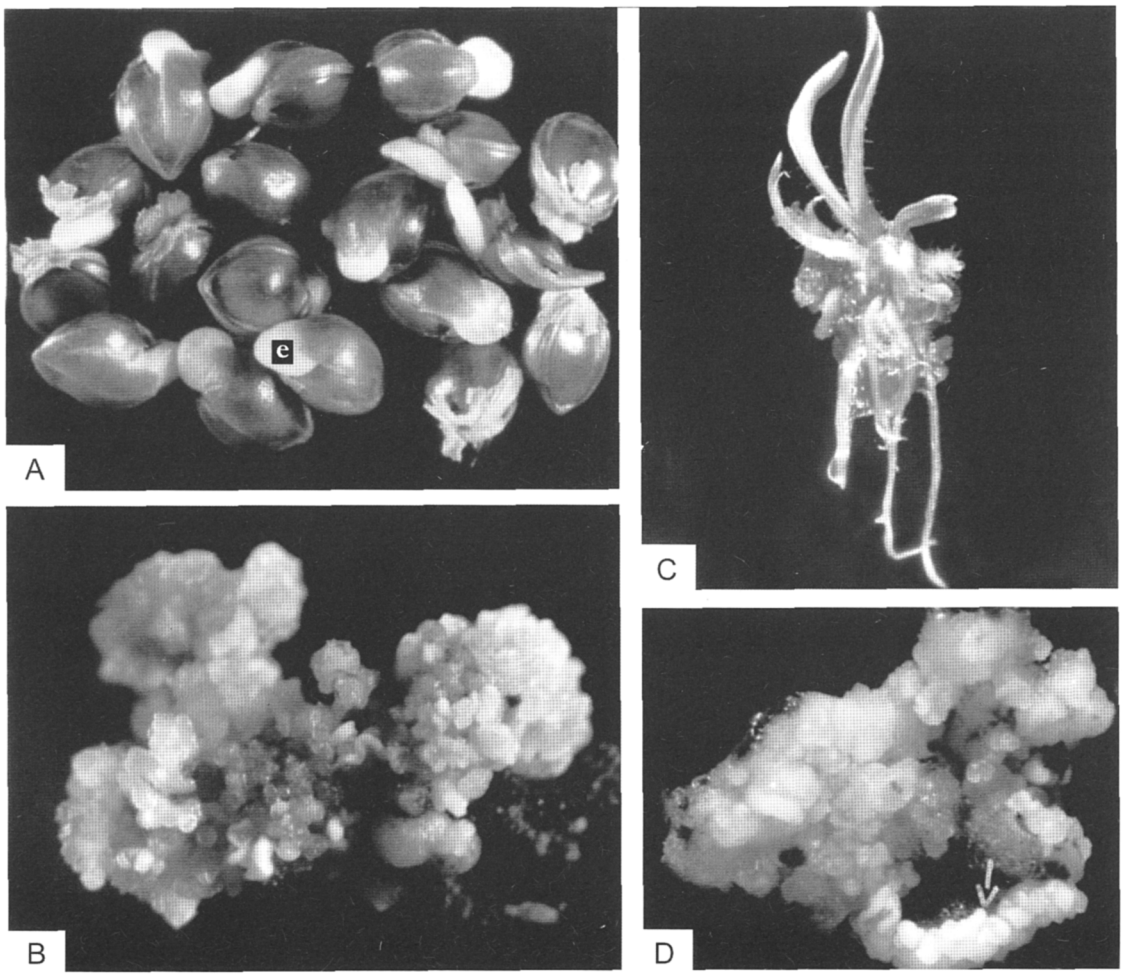

Fig. 1. *Paspalum scrobiculatum* somatic embryogenesis and organogenesis on culture of mature zygotic embryo: *A* - mature zygotic embryos (e) emerging out from soaked-seeds, prior to culture; *B* - 5-week-old culture of a mature embryo on N_6 medium with 9.0 μM 2,4-D, showing compact callus differentiating into numerous somatic embryos; *C* - 7-d treatment of a mature embryo at 100 μM 2,4-D followed by 3-week-culture on N_6 basal medium, showing differentiation of multiple-shoots and roots; *D* - 11-d treatment of mature embryo at 100 μM 2,4-D followed by 3-week-culture on N_6 basal medium, showing the formation of a compact callus and differentiation of somatic embryos (*arrow*).

Mature zygotic embryos germinated readily on 2,4-D-free basal medium. At concentrations 18.0 and 22.5 μM almost all explants exhibited direct callusing without germination. In most of these cultures two types of calli apparent were: *1*) soft and friable, white callus or *2*) hard, nodular and compact, yellow callus. In 5-week-old cultures compact calli differentiated into somatic embryos (Fig. 1*B*). These somatic embryos after transfer to hormone-free medium germinated to form plantlets.

Somatic embryogenesis was recorded in 48 % cultures (Fig. 2) on MS medium at 18.0 μM of 2,4-D whereas this frequency on N_6 medium was 53 % at 9.0 μM 2,4-D. N_6 formulation, originally suggested for anther culture of rice (Chu *et al.* 1975), was found to be superior to MS medium in support of somatic embryogenesis from inflorescence explants of *Pennisetum purpureum* (Wang and Vasil 1982) and *Pennisetum typhoides* (Talwar and Rashid 1990). In present study too, N_6 medium was relatively more effective for somatic embryogenesis than MS. The reason(s) for this remains to be resolved. These two media, differ essentially in that the former is low and the latter is high in ammonium content. Therefore, the effect of ammonium on differentiation of somatic embryos in graminaceous plants is to be resolved.

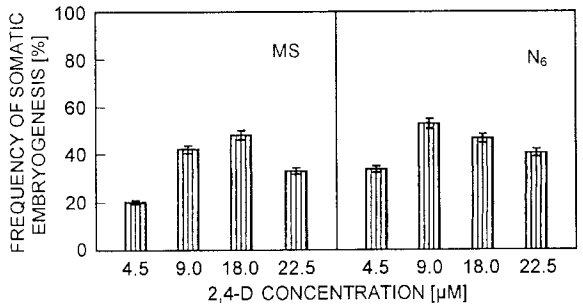

Fig. 2. Frequency of somatic embryogenesis from mature embryos of *Paspalum scrobiculatum* on MS or N_6 medium supplemented with different concentrations of 2,4-D for 5 weeks.

The embryogenic response at two different concentrations of 2,4-D (9.0 and 18.0 μM) in different media prompted us to employ 2,4-D at a higher concentration in N_6 medium, and determine the effects of different treatment periods. When explants were incubated in 100 μM 2,4-D for 2 d, almost 70 % explants germinated on transfer to hormone-free medium. Of these about 30 % explants (Fig. 3), did show some proliferation at the basal portion of the coleoptile. These young plantlets formed 2 - 3 shoots from the basal region, after 3-weeks in culture. An increase in duration of high 2,4-D treatment resulted in a significant enhancement in frequency of multiple-shoot-bearing forms (Fig. 1*C*) reaching 73 % (Fig. 3) when explants were treated for 7 d on 100 μM 2,4-D. Contrary to it embryos incubated for 11 d on 100 μM 2,4-D formed callus in almost all cultures. Of these a lower frequency (36 %) differentiated into shoot-buds on transfer to hormone-free medium whereas in 43 % cultures the calli formed differentiated into somatic embryos (Fig. 1*D*). On subjecting the explants to 100 μM 2,4-D for a longer period (up to 3 weeks), there was no morphogenic response.

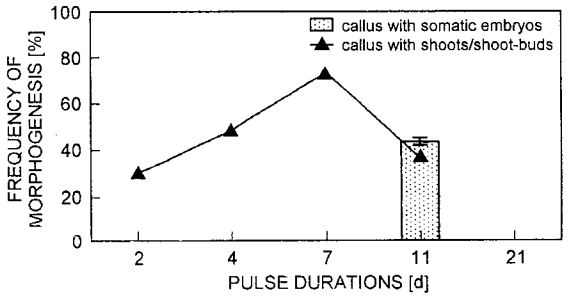

Fig. 3. Frequency of morphogenic response from mature embryos of *Paspalum scrobiculatum* pulse-treated with 100 μM 2,4-D followed by 3-week culture on hormone-free N_6 medium.

Induction of embryogenic callus and somatic embryos on culture of mature embryos of *Paspalum scrobiculatum*, on low (9.0 and 18.0 μM) 2,4-D, is in concurrence with earlier results on *P. notatum* (Bovo and Mroginsky 1989). For the establishment of an embryogenic programme in *P. scrobiculatum*, a treatment for five weeks was necessary at low concentration of 2,4-D (9.0 μM) whereas an exposure of just 11 d at 100 μM 2,4-D resulted in somatic embryogenesis. Shorter durations of 4 and 7 d resulted in differentiation of multiple-shoots. This is the first instance in monocots of a controlled regeneration response by organogenesis or embryogenesis. Somewhat similar results are described in tissue culture of maize, inbred line B-73 (Lowe *et al.* 1985). However, a distinction is desirable between the results obtained in maize and millet. Scutellum from an immature embryo of maize on culture with 2,4-D and 12 % sucrose formed a compact white callus. On subculture to medium with 2 % sucrose this callus yielded a green organogenic callus and a sector of this tissue spontaneously became embryogenic. The embryogenic callus was fast-growing, friable and mucilaginous. These cell lines, organogenic and embryogenic, have even been marked by molecular markers (Everett *et al.* 1985, Fransz *et al.* 1989) and they were of spontaneous origin. On the contrary, in *P. scrobiculatum*, it was possible to induce organogenic or embryogenic programme by keeping mature embryo explant on 2,4-D medium, for different durations. However, it remains to be resolved how shoot initiation or embryogenic response is established. Is it simply an exposure to auxin for different durations that results in organogenesis or embryogenesis, or endogenous

factor/s is/are also involved? This study also indicates that cells are not preprogrammed for a specific mode of regeneration. Their diversion towards embryogenesis or organogenesis is dependent on time of exposure to an auxin.

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