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Organogenesis and *in vitro* flowering of *Echinochloa colona*. Effect of growth regulators and explant types

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Abstract

Echinochloa colona regeneration via organogenesis in callus cultures derived from leaf base and mesocotyl explants and *in vitro* flowering were achived. Shoot bud regeneration was achieved on Murashige and Skoog's (MS) basal medium supplemented with 6.66 μM 6-benzylaminopurine (BAP), 2.68 μM 1-naphthalene acetic acid (NAA) and 3 % (m/v) saccharose. Regenerated shoots were rooted on half strength basal MS medium with 2 % (m/v) saccharose devoid of growth regulators. About 90 - 95 % of rooted plantlets survived in the greenhouse. *In vitro* flowering was induced in the regenerated shoots derived from callus on half strength MS medium supplemented with 4.4 μM BAP, 74.07 μM adeninesulphate, 0.72 μM gibberellic acid, and 3 % (m/v) saccharose. The frequency of *in vitro* flowering was 80 - 90 % in three repeated experiments. Fertile seeds were recovered from *in vitro* grown plantlets which were subsequently germinated into plants.

Additional key words: metal tolerance, regeneration.

Introduction

Echinochloa colona (L.) Link is an annual grass commonly found in association with wetland paddy and other crops on irrigated uplands. Grain of *Echinochloa colona* is used as food by impoverished people; it is also much valued as a fodder. It has been reported to be tolerant to chromium, nickel and iron and hence, offers to provide an effective cover on minewaste dumps (Samantaray 1991). *In vitro* flowering has been reported earlier in monocots as bamboo (Rout and Das 1994) and pearl millet (Kulkarni *et al.* 1995). The present investigation deals with the effect of growth

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Abbreviations: Ads - adeninesulphate; BAP - 6-benzylaminopurine; GA_3 - gibberellic acid; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; Kn - kinetin; NAA-1-naphtaleneacetic acid; MS - Murashige and Skoog.

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regulators on plant regeneration via organogenesis and *in vitro* flowering of *Echinochloa colona* with useful genetic traits.

Materials and methods

Explant source: Mature seeds of *Echinochloa colona* (L.) Link were collected from a naturally growing plant population on minewaste dumps of an open-cast chromite mine in a monsoon climate at Sukinda, India. The seeds were washed in 2 % (v/v) detergent solution *Teepol* (*Qualigen*, Bombay, India) and surface sterilized in 0.1 % (m/v) aqueous $HgCl_2$ solution for 15 min and rinsed five times with sterile distilled water. An average of 10 - 12 seeds were aseptically cultured in 25 × 150 mm glass tubes (*Borosil*, Bombay, India) containing 25 cm^3 of the medium consisting of Murashige and Skoog (MS) (1962) mineral salts and 555 µM *m*-inositol, 4.06 µM nicotinic acid, 2.43 µM pyridoxine-HCl, 0.296 µM thiamine-HCl, 2 % (m/v) saccharose and 0.8 % (m/v) agar (*Qualigen*, Bombay). The medium was without phytohormone and tubes were capped with non-absorbent cotton plugs wrapped in one layer of cheese-cloth. The pH was adjusted to 5.8 with 0.1 M HCl or 0.1 M NaOH before adding agar and autoclaving at 121 °C and 104 kPa for 15 min. The cultures were incubated under 16 h photoperiod (cool-white fluorescent light, irradiance 50 µmol m^{-2} s^{-1}) at temperature 25 ± 2 °C and relative humidity 60 %. The preliminary experiments showed that 10-d-old seedlings exhibited a morphogenic response (data not shown) and, thus, were utilized in subsequent experiments. Leaf bases (3 - 5 mm) and mesocotyls (4 - 5 mm) derived from 10-d-old seedlings were used as explants.

***In vitro* culture:** For induction of callus and organogenesis, all cultures were maintained on MS basal medium supplemented with different concentrations and combinations of BAP (0.0, 1.11, 2.22, 4.44, 6.66 and 8.88 µM), Kn (0.0, 1.16, 2.32, 4.64, 6.96 and 9.28 µM), IAA (0.0, 2.85, 5.71, 8.57, 11.42 and 14.28 µM), IBA (0.0, 2.46, 4.92, 7.38, 9.84 and 12.30 µM) and NAA (0.0, 0.53, 1.34, 2.7 and 5.37 µM). The media pH was adjusted to 5.8 using 0.1 M HCl or 0.1 M NaOH before autoclaving. Routinely, 25 cm^3 of the molten medium gelled with 0.8 % (m/v) agar was dispensed into culture tubes (25 × 150 mm) and plugged with non-absorbent cotton wrapped in one layer of cheese cloth. The culture tubes were then steam sterilised at 121 °C at 104 kPa for 15 min.

Mesocotyl and leaf base explants (2 - 3) were inoculated into separate culture tubes (25 × 150 mm). Incubation conditions were the same as for germination of seeds. Each treatment consisted of 25 replicates per treatment and each experiment was repeated three times. Visual observations of the cultures were taken every week and the effects of different treatments were quantified on the basis of the percentage of culture showing response. The number of regenerants per culture was recorded after 6 weeks of initial incubation. The cultures were subcultured at 6-week intervals into fresh medium of the same composition.

***In vitro* flowering:** The regenerated shoots derived from leaf base and mesocotyl callus were cultured for induction of multiple shoots. The shoots were grown on half-strength basal semi-solid MS medium supplemented with 4.4 µM BAP, 74.07 µM Ads, 0.72 µM GA_3 and 3 % (m/v) saccharose for induction of flowering. Cultures were grown in 250 cm^3 conical flasks (*Borosil*, Bombay, India) with 100 cm^3 nutrient medium per flask and incubated at 25 ± 2 °C in a growth room under 16 h photoperiod.

Induction of rooting from regenerated shoot and transplantation: *In vitro* raised shoots measuring 3 - 4 cm were separated and cultured on half-strength MS medium without phytohormones. Rooted micropropagules were throughly washed to remove the adhering gel before transfer to 2.5 cm clay pots containing minewastes; transplants were kept in a humidified chamber for better establishment. The plants flowered and produced seeds in these conditions.

Results

Callus induction: Callus was initiated both from leaf base and mesocotyl segments derived from 10-d-old *in vitro* grown seedlings of *Echinochloa colona* on MS basal medium supplemented with various concentrations and combinations of BAP and NAA after 4 weeks of incubation. The maximum callus proliferation was noted in the medium containing 2.22 µM BAP and 10.74 µM NAA (Fig. 1A); the rate of callus proliferation was more in leaf base than mesocotyl explants (Table 1). The other auxins (IAA and IBA) tested were not as an efficient to induce callusing (data not shown). The quality of callus was better with BAP compared to Kn; the callus was creamy white and friable. The optimal concentration of BAP and NAA for callus induction did not vary between the explant types. When the concentration of NAA or BAP was increased, the rate of callus growth decreased and the calli became compact.

Table 1. Effect of different concentrations of BAP and NAA on callus induction (mean percentage of explant callusing, 25 replicates per culture) from mesocotyl (M) and leaf base (LB) explants of *Echinocloa colona* after 6-weeks cultivation.

BAP [µM]	0.0		2.22		4.44		8.88	
NAA [µM]	M	LB	M	LB	M	LB	M	LB
0.0	0	0	0	0	0	0	0	0
2.7	10.3±0.2	16.4±0.4	20.4±0.5	22.6±0.6	18.2±0.5	23.3±0.4	20.4±0.4	24.2±0.6
5.4	14.4±0.4	22.2±0.3	32.2±0.1	41.2±0.5	28.4±0.4	32.2±0.6	23.2±0.6	28.4±0.3
8.0	32.2±0.1	36.4±0.2	43.1±0.5	53.8±0.4	32.4±0.2	34.4±0.6	32.6±0.4	34.2±0.8
10.7	38.4±0.4	47 1±0.4	61.6±0.8	76.4±0.2	38.6±0.6	42.2±0.8	38.6±0.7	42.4±0.5
13.4	42.4±0.4	56.7±0.6	57.2±0.4	67.2±0.3	44.1±0.3	51.2±0.2	37.2±0.3	36.6±0.2
16 1	52.3±0.2	62.4±0.1	48.4±0.3	52.4±0.8	54 2±0.5	60.2±0.6	42.3±0.8	36.8±0.1

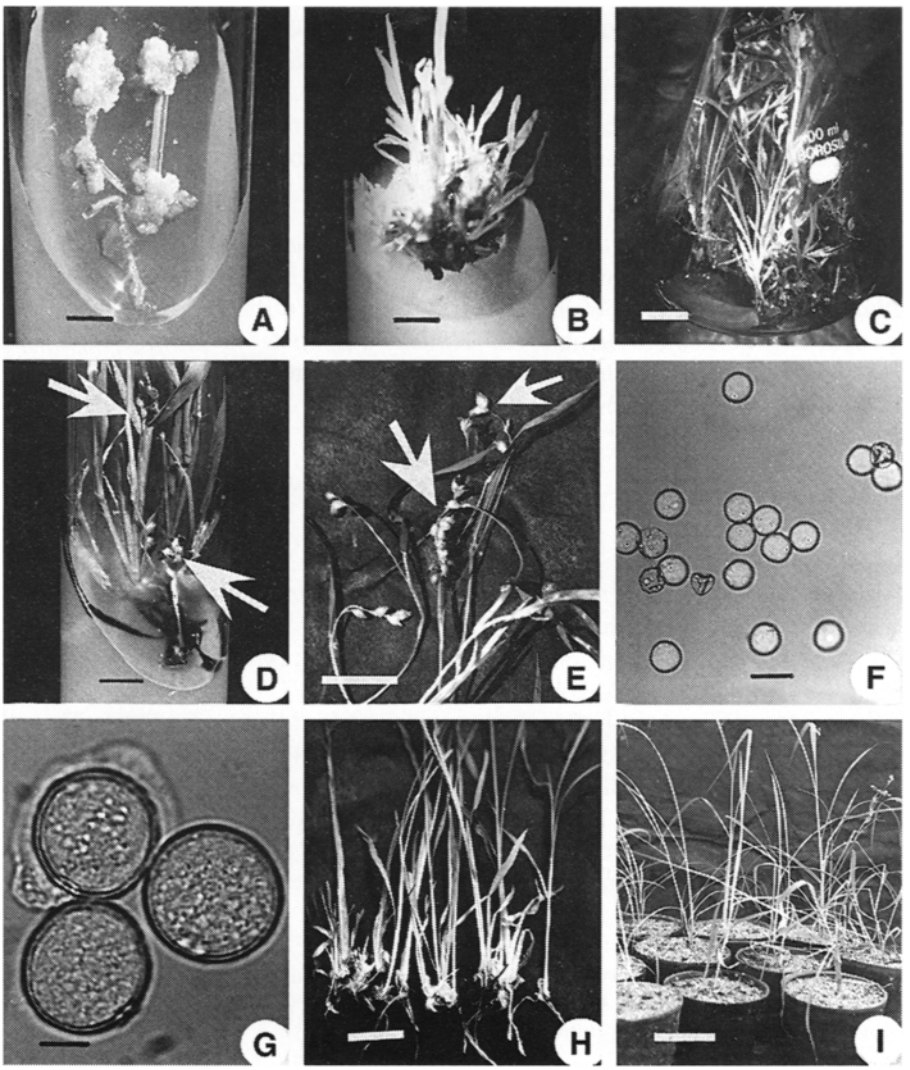
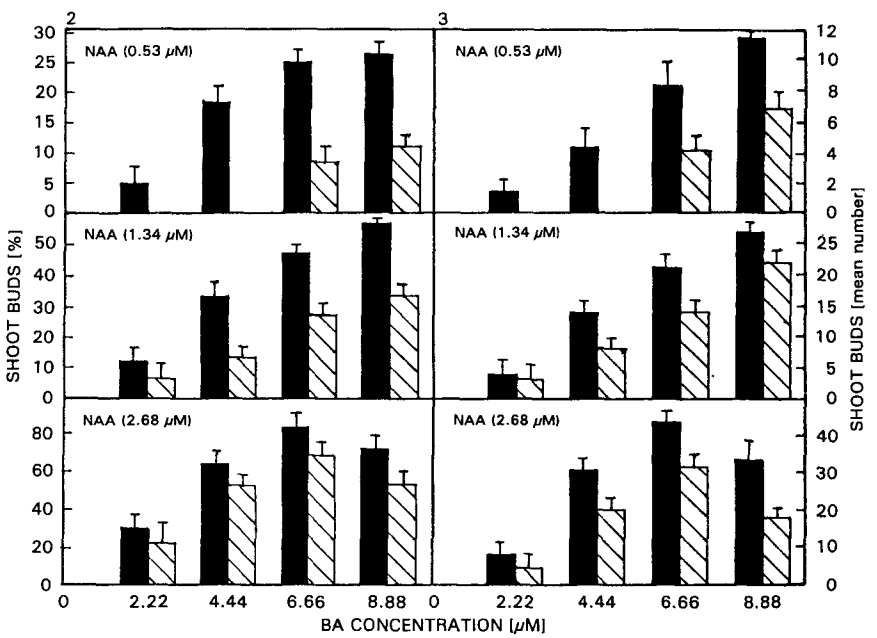


Fig. 1. *In vitro* plant regeneration and flowering of *Echinochloa colona* (L.) Link.: *A* - induction of callus from leaf base explants cultured on MS medium supplemented with 2.22 μM BAP + 10.74 μM NAA after 3 weeks of culture (*bar* = 0.2 cm); *B, C* - regeneration of shoot buds from leaf base calli cultivated on 6.66 μM BAP + 2.68 μM NAA after 3 (*B*) or 6 (*C*) weeks of culture (*bar* = 0.2 and 0.5 mm for B and C, respectively); *D* - *in vitro* flowering (*arrows*) of *Echinocloa colona* after 4 weeks of culture on 4.44 μM BAP, 74.07 μM Ads, 0.72 μM GA_3 and 30 g dm^{-3} saccharose (*bar* = 0.2 mm); *E* - peduncle with 4 - 6 flower buds (*arrows*) (*bar* = 0.5 mm); *F, G* - pollen grains of *in vitro* flowering plantlets (*bar* = 0.1 and 0.2 mm for *F* and *G*, respectively); *H* - regenerated shoots were rooted after 10 - 12 d of culture on 1/2 MS medium without growth regulators (*bar* = 0.25 cm); *I* - plantlets growing in earthen pots (*bar* = 0.2 cm).

Regeneration of shoot buds: Both mesocotyl and leaf base derived calli transferred to the regeneration medium (MS + 6.66 µM BA, 2.68 µM NAA and 3 % saccharose), gave rise to greenishwhite shoot buds after 3 weeks, which subsequently developed into shoots (Figs. 1B and C). Supplementation of MS medium with Kn (1.16 - 9.28 µM) or BAP (1.11 - 8.88 µM) alone or in combination did not show any response even when cultured for a prolonged period. The explants cultured on medium containing NAA (0.53 - 8.05 µM) alone or in combination with Kn at different concentrations failed to induce shoot buds. The medium containing BAP (4.44, 6.66 and 8.88 µM) with NAA (0.53, 1.34 and 2.68 µM) induced regeneration of shoot buds on mesocotyl explants; however, on leaf base explants shoot buds developed in the medium containing both BAP (4.44, 6.66 and 8.88 µM) and NAA (1.34 and 2.68 µM). The frequency of shoot bud regeneration was the maximum with BAP (6.66 µM) in combination with NAA (2.68 µM) (Fig. 2); higher concentrations of NAA (5.37 µM) failed to induce shoots. The number of shoot buds per culture varied significantly between (1.34 ± 0.2 to 44.24 ± 0.3) in mesocotyl and (8.32 ± 0.2 to 32.16 ± 0.3) in leaf base explants, respectively (Fig. 3). Calli grown on high auxin medium when transferred to the regeneration medium (different combination of auxin and cytokinin) did not elicit any positive effect on plant regeneration. In 20 % of the cultures, the initial shoots became albions which turned green at the later stages of growth.



Figs. 2 and 3. Effect of various concentrations of BAP and NAA on percentage of shoot regeneration (Fig. 2) and average number of shoots per culture (Fig. 3) from calli derived from mesocotyl and leaf base explants of *Echinochloa colona* after 6 weeks of culture (25 replicates per each treatment, repeated thrice; shoots less than 0.5 cm were not counted).

***In vitro* flowering:** The regenerated shoots were isolated and cultured on medium containing different combinations of auxin and cytokinin for *in vitro* flowering. The shoots flowered after 4 weeks of subculture in the medium containing 1/2 strength MS medium supplemented with 4.4 μM BAP, 74.07 μM Ads, 0.72 μM GA_3, and 3 % saccharose (Fig. 1D). About 60 - 70 % of the plantlets produced panicles (1 to 2 mm). The internodes of the rachis were highly condensed. The flower buds were borne on a thin peduncle with only 4 - 6 buds of normal size (1.2 mm) (Fig. 1E). Violet flowers opened 2 - 3 d after emergence of the panicle. This protogynous pattern of flowering was similar to that of the field grown plants but the time from panicle emergence to opening of flower was considerably shortened, 2 - 3 d under *in vitro* condition as compared to 7 d in the open air. Only 75 % of pollen grains were fertile (as revealed by iodine staining), others were empty or partially filled, whereas in case of field grown plants more than 90 % of the pollen grains were fertile (Figs. 1F and G). About 20 - 25 viable seeds were obtained from each culture.

Rooting of regenerated shoots: Regenerated shoots developed roots on induction medium having half strength MS basal nutrients without vitamins and growth regulators. The *in vitro* derived shoots developed roots profusely, about 90 % of regenerated shoots rooted within 10 to 12 d (Fig. 1H).

Transfer of plantlets to pots: The rooted plantlets were transferred to pots containing autoclaved soil mixture (soil:sand:manure) at the ratio of 1:1:1 and were maintained in a greenhouse (Fig. 1I). The plantlets grew normally, flowered and set seeds.

Discussion

The present studies have shown that it is possible to induce plant regeneration and *in vitro* flowering from callus derived from leaf base and mesocotyl segments of *Echinochloa colona*. The use of various explants for the production of organogenic calli and subsequently plant regeneration of some cereals and grasses is well documented (Terada *et al.* 1987, Wang *et al.* 1987, Rueb *et al.* 1994).

In *Echinochloa colona*, NAA was found to be suitable for the induction of plant regeneration; IBA and IAA were not effective in the induction of shoot buds at the concentration tested. NAA has been widely used for plant regeneration in various cereals and grasses (Eapen and Rao 1985, Nabors *et al.* 1983). Specific micro-environmental conditions were required to sustain regenerative capacity of callus derived from various explants of grass species (Cure and Mott 1978, O'Hara and Street 1978). Our results demonstrated that, sequential transfer of developing callus from leaf base and mesocotyl explants to media with reduced NAA concentration was required to achieve plantlet regeneration. The ability of different type of explants to develop shoot buds have been well documented in grasses (Conger 1981, Conger and Carabia 1978, Finch *et al.* 1992 and Dale and Dembrogio 1979) and in *Oryza sativa* (Nakano and Maeda 1979). The regenerated shoot buds rapidly multiplied and elongated on similar induction medium; the rate of multiple shoot formation, however, depended on growth regulators as reported earlier in monocots

and grasses (Vasil 1982, Wang and Vasil 1982, Finch *et al.* 1992). In some of the cultures albinos developed which turned green at the later stages of growth. Similar results were reported earlier in bamboo (Rout and Das 1994). This system for efficient regeneration of *Echinochloa colona* from callus could be applied to improve cultivar selection after environmental stress testing at the cellular level. The regenerated shoots flowered after 4-week of subculture in the 1/2 MS culture medium containing 4.4 µM BAP + 74.07 µM Ads + 0.72 µM GA_3 and 3 % saccharose. About 40 % of the flowers giving fertile seeds was considered to be normal under these growth conditions. About 20 - 25 viable seeds were obtained from each culture. The *in vitro* flowering with viable seeds in monocot was reported earlier in bamboo (Rout and Das 1994), rice (Rueb *et al.* 1994) and *Pennisetum glaucum* (Kulkarni *et al.* 1995). The regenerated shoots were rooted in half-strength MS basal nutrients without vitamins and growth regulators. About 90 - 95 % of the rooted plantlets survived under the field conditions, grew normally, flowered and set seeds.

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