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BRIEF COMMUNICATION

Improvement of *in vitro* androgenesis in niger using amino acids and polyamines

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Abstract

The effects of amino acids (arginine, aspargine, cysteine, glutamine, glycine and proline) and polyamines (putrescine and spermidine) on embryogenesis and plant regeneration from cultured anthers of *Guizotia abyssinica* (L. f.) Cass. cv. Ootacamund was studied. Supplementation of amino acids (0.5 - 5.0 mM) to the induction medium individually and in combination, improved embryo yield. B5 medium supplemented with 2 mM proline, 10 μM 2,4-dichlorophenoxy-acetic acid , 2 μM kinetin and 0.2 M sucrose induced highest number of embryos (63 per 60 anthers cultured). Addition of polyamines (5 - 200 μM) to the same medium also enhanced the rate of embryogenesis.

Additional key words: anther culture, embryogenesis, *Guizotia abyssinica*.

Niger is an oilseed crop cultivated in India, Ethiopia, Kenya, Uganda and Malawi. Niger seed yields 35 - 40 % oil, which is edible, and it is also used in manufacturing soaps, cosmetics, and paints and for lubrication. Self-incompatibility of niger hinders the production of homozygous lines (Getinet and Sharma 1996). Homozygous lines obtained through anther and microspore culture would be used for hybridization and crop improvement. Induction of embryogenesis from cultured anthers has been reported in niger (Survesh *et al.* 1993), however, the frequency of embryo formation was meagre. Generally, medium supplemented with growth regulators such as auxins, cytokinins, gibberellins and abscisic acid have been used for induction of embryogenesis/ organogenesis and plantlet regeneration from cultured anthers in many species (Bajaj 1990). Amino acids such as glutamine for *Triticum aestivum* (Indrianto *et al.* 1999), serine for *Brassica oleracea* (Dias and Martins 1999), thiamine and glycine for *Oryza sativa* (Guzman and Zapata 2000) supplemented to anther culture medium have shown positive effect. The addition of an amino acids mixture enhanced androgenesis in *Hordeum vulgare* (Ouédraogo *et al.* 1998) and *Cucumis sativus* (Ashok Kumar and Murthy 2004). Supplementation of polyamines to culture medium has triggered the process of somatic embryogenesis in oat (Kelly *et al.* 2002), ginseng (Kevers *et al.* 2000, Monteiro *et al.* 2002), oil palm (Rajesh *et al.* 2003) and grape (Bertoldi *et al.* 2004). In *Solanum tuberosum* (Tiainen 1992) and *Cucumis sativus* (Ashok Kumar *et al.* 2004), spermidine and putrescine significantly increased embryos yield in cultured anthers. The main objective of the present study was to examine the influence of amino acids (arginine, aspargine, cysteine, glutamine, glycine and proline) and polyamines (putrescine and spermidine) on androgenesis of niger.

Niger [*Guizotia abyssinica* (L. f.) Cass. Asteraceae] cv. Ootacamund seeds were obtained from Indian Council of Agriculture research, Jawaharlal Nehru Agricultural University Campus, Jabalpur, Madhya Pradesh, India. Plants were grown in the experimental plots at Department of Botany, Karnatak University, Dharwad, Karnataka, India using standard agronomic practices. The immature capitula were collected when most of the microspores were at the uni-nucleate stage and stored at 4 °C for 3 d in dark. Pretreated capitula

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Abbreviations: ABA - abscisic acid; BA - N^6- benzyladenine; B5 - Gamborg *et al*. (1968) medium; 2,4-D - 2,4-dichlorophenoxy-acetic acid; KN - kinetin.

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were washed with 1 % (v/v) *Laboline* (detergent) and 0.5 % *Carbendazim* (fungicide) for 10 min and surface sterilized with 5 % sodium hypochlorite solution for 20 min in orbital shaker at 100 rpm. The final step of sterilization was carried out in a horizontal laminar air flow chamber by rinsing the capitula twice in sterile distilled water, followed by 0.1 % mercuric chloride solution for 5 min. Finally, the capitula were rinsed five times in sterile distilled water. Anthers were isolated aseptically from florets and cultured onto an embryo induction medium. Auxins, cytokinins, amino acids, abscisic acid and polyamines were purchased from *Sigma Chemical Company*, St. Louis, USA, and membrane filters were purchased from *Millipore Company*, Bangalore, India. *Carbendazim* was procured from *Hyderabad Chemical Supplies Limited*, Hyderabad, India.

The embryo induction medium was B5 medium (Gamborg *et al*. 1968) supplemented with 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 2 μM kinetin (KN) and 0.2 M sucrose. Amino acids such as arginine, aspargine, cysteine, glutamine, glycine and proline at concentration 0.5, 1.0, 2.0, 5.0 mM and a combination of them at 1 mM and 2 mM concentration were amended to above mentioned medium. Similarly, putrescine and spermidine at 5, 10, 50, 100, 200, 500 and 1000 μM were added to this medium. For differentiation of embryos, the globular stage embryos were subcultured to B5 medium supplemented with 0.5 μM N^6-benzyladenine (BA) and 0.09 M sucrose. For maturation of embryos, the torpedo stage embryos were cultured onto B5 medium supplemented with 10 μM abscisic acid (ABA). Mature embryos were isolated aseptically and cultured onto B5 medium containing 0.09 M sucrose. The pH of all the media was adjusted to 5.8 using 0.1 M NaOH or HCl and was solidified with 0.8 % agar (*Himedia*, Mumbai, India). Media were sterilized by autoclaving at 120 °C for 20 min. Vitamins, amino acids, polyamines and ABA were filtered using sterilized membrane filters (0.45 μm; *Millipore*, Bangalore, India) and added to the autoclaved medium.

The cultures were kept in the dark at 24 ± 2 °C for two weeks and further incubation was at 24 ± 2 °C and 16-h photoperiod with irradiance of 40 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes. Well-developed plantlets were removed from cultures and washed in sterilized distilled water to remove the traces of media. These plantlets were transplanted to plastic cups containing a mixture of autoclaved *Vermiculite*, sand and garden soil (1:1:1). The transplanted plantlets were acclimatized in a plant growth chamber for 15 d under relative humidity 80 %, temperature 24 ± 2 °C, irradiance 40 μmol m^{-2} s^{-1} and 16-h photoperiod before transferring to greenhouse for further growth.

Embryos at different developmental stages were fixed in FAA (formalin: glacial acetic acid: 70 % ethanol, 10:5:85) for 12 h at room temperature, dehydrated through a graded ethanol-butyl alcohol series and embedded in paraffin wax (Fowke and Rennie 1996). The tissues sectioned at a thickness of 6 μm were stained with 0.05 % toluidine-blue solution and examined under compound microscope.

The experiments were arranged in a complete randomized design with 12 replicates in each experiment and each replicate consisting of 4 - 6 anthers. Each experiment was repeated three times. The cultures were observed periodically and morphological changes were recorded at weekly intervals and subjected to analysis of variance (*ANOVA*) and mean values were separated according to Duncan's multiple range test (DMRT).

Callus mediated embryogenesis were observed from the cultured anthers of niger, anthers swell in two weeks and turned brown (Fig. 1*A*) and induced callus in another two weeks (Fig. 1*B*). Globular embryos were formed from the embryonic callus in two weeks (Fig. 1*C*). All the amino acids (arginine, aspergine, cystine, glutamine, glycine and proline; 0.5 - 5 mM) amended individually to induction medium improved the embryogenic response of anthers and the yield of embryos (Table 1). Among the six amino acids tested proline induced greater number of embryos followed by glycine, aspargine, cystine, glutamine and arginine. The highest number of embryos

Table 1. Effect of different amino acids (or their combination) supplemented to B5 medium with 10 μM 2, 4-D, 2 μM KN and 0.2 M sucrose on anther culture of niger cv. Ootacamund. 60 anthers were cultured. In each column, mean values followed by same letters are not significantly different according to DMRT at $P = 0.05$.

Amino acid	Conc. [mM]	Responding anthers [%]	Number of embryos	Regeneration [%]
Control	0.0	32	45 s	30
Arginine	0.5	37	48 qr	28
	1.0	39	51 mn	29
	2.0	43	54 ij	31
	5.0	40	51 lm	32
Aspergine	0.5	34	50 no	33
	1.0	38	55 gh	35
	2.0	43	59 cd	40
	5.0	41	53 k	28
Cysteine	0.5	32	48 pq	23
	1.0	37	51 lm	27
	2.0	40	55 hi	31
	5.0	38	52 lm	26
Glutamine	0.5	41	50 op	32
	1.0	44	52 kl	32
	2.0	47	51 lm	32
	5.0	44	50 no	30
Glycine	0.5	41	52 kl	29
	1.0	43	56 fg	35
	2.0	49	61 c	41
	5.0	46	58 e	38
Proline	0.5	47	54 hi	29
	1.0	51	60 cd	39
	2.0	54	63 a	41
	5.0	49	60 c	36
Combination	1.0 each	52	61 b	38
Combination	2.0 each	48	56 f	36

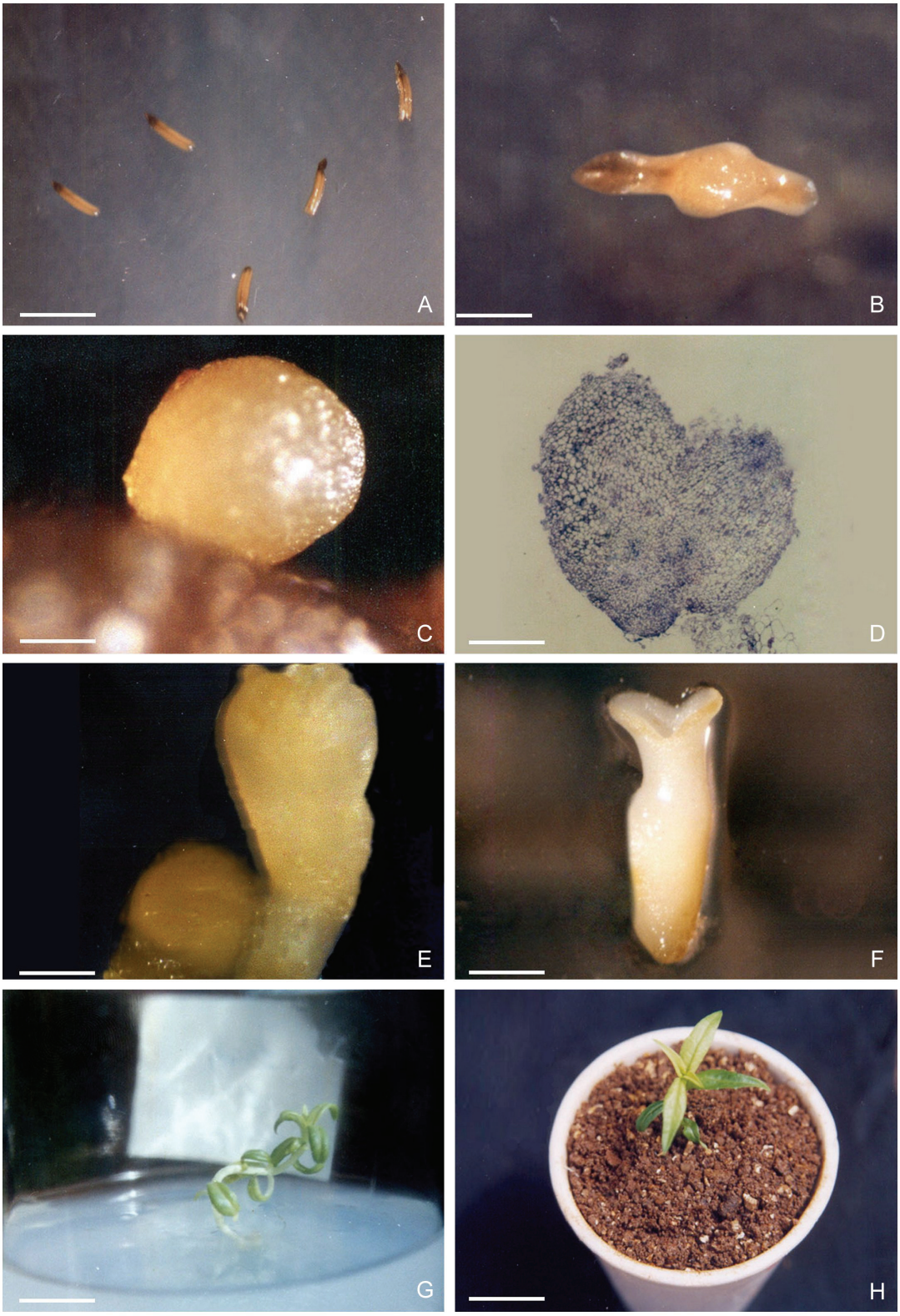

Fig. 1. Embryogenesis and plant regeneration in cultured anthers of niger cv. Ootacamund. *A* - Brown turned anthers after two weeks of culture on induction medium (*bar* = 0.2 mm). *B* - Emergence of microcalli from the anther after four weeks of culture (*bar* = 0.5 mm). *C* - Globular embryo originating from the embryogenic calli (*bar* = 0.8 mm). *D* - Longitudinal section of the heart shaped embryo (*bar* = 0.5 mm). *E* - Torpedo shaped embryo (*bar* = 1mm). *F* - Cotyledonary stage embryo (*bar* = 2 mm). *G* - An anther derived plantlet on embryo germination medium (*bar* = 1.4 cm). *H* - Transplanted plantlet (*bar* = 5 cm).

(60 and 63) was induced from 51 and 54 % responding anthers on medium supplemented with 1.0 and 2.0 mM proline in six to eight weeks. Similar to the present results Büter *et al*. (1991) has reported the positive role of proline in induction of embryogenesis from anthers of triticale and maize respectively.

Table 2. Effect of polyamines on induction of embryogenesis from cultured anthers of niger cv. Ootacamund on B5 medium supplemented with 10 μM 2, 4-D, 2 μM KN and 0.2 M sucrose. 60 anthers were cultured. In each column, mean values followed by same letters are not significantly different according to DMRT at $P = 0.05$.

Polyamine	Conc. [μM]	Responding anthers [%]	Number of embryos	Regeneration [%]
Control	0	38	40 i	12
Putrescine	5	39	47 g	14
	10	41	50 f	15
	50	43	54 cd	17
	100	46	57 a	18
	200	45	56 ab	17
	500	44	54 bc	14
	1000	41	50 f	13
Spermidine	5	39	46 gh	14
	10	42	50 f	15
	50	44	52 e	16
	100	44	54 bc	17
	200	47	57 a	18
	500	46	55 ab	17
	1000	44	53 e	15

Addition of a combination of amino acids (arginine, aspargine, cystine, glutamine, glycine and proline; 1.0 and 2.0 mM) to the induction medium was beneficial in induction of embryos (Table 1). Addition of a combination of amino acids has also been beneficial for induction of embryos and plantlet regeneration in *Cucumis sativus* (Ashok Kumar and Murthy 2004), *Hordeum vulgare* (Ouédraogo *et al.* 1998), *Triticum aestivum* (Trottier *et al.* 1993), and *Zea mays* (Claparols *et al.* 1993). Embryo induction was also improved with the addition of putrescine and spermidine (5, 10, 50, 100, 200, 500, 1000 μM) to the induction medium compared to the control (Table 2). Among the different concentrations of putrescine, optimum of 57 embryos developed from 60 anthers cultured on medium supplemented with 100 μM putrescine. Putrecine is an important modulator of biological processes such as cell division, growth and differentiation (Walden *et al.* 1997) and embryogenesis was improved with addition of putrescine to embryo induction medium (Kelly *et al.* 2002, Ashok Kumar *et al.* 2004).

Addition of spermidine to embryo induction medium was shown to increase embryogenic process in potato (Tiainen 1992), ginseng (Monteiro *et al.* 2002) and cucumber (Ashok Kumar *et al.* 2004). In the present study, maximum of 57 embryos were produced from 60 anthers on embryo induction medium supplemented with 200 μM spermidine.

Embryos induced on different induction media were differentiated in to heart and torpedo shaped embryos (Fig. 1*D,E*) in four weeks upon subculturing globular embryos on-to differentiation medium. ABA treatment was necessary for maturation and subsequent development of somatic embryos in many crop plants (Bajaj 1990) and to improve plantlet development in androgenesis (Palmer and Keller 1997). In the present studies torpedo shaped embryos were transferred to maturation medium containing 10 μM ABA upon which embryos matured into cotyledonary embryos within two weeks (Fig. 1*F*). Mature embryos germinated into plantlets in another two weeks on B5 medium supplemented with 0.09 M sucrose (Fig. 1*G*). The percentage of plantlet regeneration was also high with 41 % of survival from the embryos induced on media supplemented with 2 mM proline (Table 1) whereas 18 % of plantlets regeneration was observed from the embryos, which were induced on medium supplemented with 100 μM putrescine and 200 μM spermidine.

The plantlets were transplanted to potting mixture of *Vermiculite*, sand, garden soil (Fig. 1*H*) and reared in growth chamber in controlled environmental conditions. Thirty-four plantlets survived out of sixty transplanted plants. Twelve plants showed the haploid chromosome number (n=15) and the remaining were diploids. From the present investigation it can be concluded that the efficiency of embryogenesis and plantlet regeneration from cultured anthers of niger was increased with the addition of amino acids and polyamines to the induction medium.

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