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BRIEF COMMUNICATION

The effect of sugars on niger embryogenesis and plant regeneration in anther culture

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Abstract

The influence of different sugars (sucrose, maltose, glucose and fructose, 0.05 - 0.5 M) on embryogenesis and plant regeneration from cultured anthers of niger [*Guizotia abyssinica* (L. f.) Cass.] have been studied. Among the different sugars tested, 0.2 M sucrose was the best for embryo induction and plant regeneration. Maximum of 57 embryos per 60 anthers were induced on embryo induction medium [Gamborg's B5 medium supplemented with 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 2 μM kinetin (KIN)] containing 0.2 M sucrose. Embryo differentiation was achieved on B5 medium supplemented with 0.5 μM benzyladenine (BA) and 0.09 M sucrose. Embryo maturation was on B5 medium containing 10 μM abscisic acid (ABA) and 0.09 M sucrose. Embryo germination was achieved on B5 medium with 0.09 M sucrose. Embryos that were developed on B5 medium supplemented with 0.2 M sucrose showed highest frequency (68 %) of plant regeneration.

Additional key words: fructose, glucose, growth regulators, *Guizotia abyssinica*, haploids, maltose, sucrose.

Niger [*Guizotia abyssinica* (L.f.) Cass.] is an oil seed crop plant belonging to family *Asteraceae*. It is grown extensively in the Indian subcontinent and East African countries for seeds containing about 30 - 40 % of a yellow, edible oil. Self-incompatibility nature of niger complicates the production of inbred line development and maintenance (Getinet and Sharma 1996). Alternative method for homozygous line production is by induction of haploids by anther culture and subsequent production of double haploids (Bajaj 1990).

Among the various factors that influence the androgenesis, culture medium and/or its components are most important once. Sugar is the source of carbon and energy and also acts as an osmotic regulator in the induction medium (Ferrie *et al.* 1995). The type and concentration of sugars in the medium has been found to influence induction of embryogenesis from cultured anthers (Ashok Kumar and Murthy 2004, Ferrie *et al*. 1995). Sucrose is the most widely used sugar and 0.09 M sucrose for *Quercus suber* (Bueno *et al*. 1997), 0.3 M for *Avena sativa* and *A. sterilis* (Kiviharju and Pehu 1998), 0.44 M for *Cucurbita pepo* (Metwally *et al*. 1998), 0.18 M for *Oryza sativa* (Afza *et al*. 2000), and 0.25 M for *Cucumis sativus* (Ashok Kumar and Murthy 2004) have shown to enhance embryogenesis. For *Secale cereale* (Immonen and Anttila 2000) maltose was used as carbon source while for strawberry (Owen and Miller 1996) glucose and for *Triticum aestivum* (Chu *et al*. 1990) fructose proved to be the best carbon source.

Induction of embryogenesis from cultured anthers and plantlet regeneration has been reported in niger (Murthy *et al*. 2000, Sarvesh *et al.* 1993). However, the frequency of embryo production and plant regeneration was very low. Therefore, in the present investigation we have verified the effect of sugars on induction of embryogenesis and plant regeneration from the cultured anthers of niger. The overall objective was to enhance the embryo induction and plantlet regeneration in this species.

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Abbreviations: BA - N^6-benzyladenine; B5 - Gamborg's medium; 2,4-D - 2,4-dichlorophenoxyacetic acid; KIN - kinetin; ABA - abscisic acid.

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Guizotia abyssinica (L. f.) Cass. cv. Ootacamund plants were grown in the experimental plots using standard agronomic practices. Immature capitula with whitish green florets having microspores at mid to late uni-nucleate stage were collected from 30 to 40-d-old healthy plants around 09:00 to 10:00 on sunny days. The stage of microspores was determined by microscopic observation of anthers stained with 1 % acetocarmine. The capitula were collected in a conical flask and kept in a beaker containing 10 cm^3 of tap water. The mouth of the beaker was tightly wrapped with aluminum foil to maintain a high humidity and kept in dark at 4 °C for 3 d. Before culturing of anthers, capitula were washed in 1 % (v/v) *Laboline* (detergent) and 0.5 % *Carbendazim* (fungicide) for 10 min and surface sterilized with 5 % sodium hypochlorite solution for 20 min. The final step of sterilization was carried out in a horizontal laminar airflow bench using 0.1 % mercuric chloride solution for 5 min. Finally, the capitula were rinsed several times in sterile distilled water. Anthers were removed from flower buds and inoculated onto an embryo induction medium.

The medium employed for anther culture was B5 medium (Gamborg *et al*. 1968) containing 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2 μM kinetin (KIN). Sucrose, maltose, glucose and fructose were added individually at 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50 M to induction medium to verify the suitability and concentration of sugars. The responding cultures (embryogenic calli) were subcultured to B5 medium supplemented with 0.5 μM BA and 0.09 M sucrose for embryo differentiation. Torpedo and cotyledonary embryos were cultured on B5 medium containing 10 μM ABA and 0.09 M sucrose for embryo maturation. For embryo germination, mature embryos were cultured onto B5 medium containing 0.09 M sucrose. The pH of the media was adjusted to 5.8 and solidified with 0.8 % agar (*Himedia*, Mumbai, India). Medium was dispensed into Petri dishes, rimless culture tubes (15 × 2.5 cm) or 100-cm^3 conical flasks (*Borosil* (Mumbai, India). Culture tubes and flasks were plugged with non-absorbent cotton wrapped with cheesecloth or autoclavable caps. The Petri dishes, culture tubes and culture flasks were sterilized by autoclaving at 120 °C for 20 min. Thermolabile compounds such as vitamins, amino acids and ABA were filtered using membrane filters (*Millipore*; 0.45 μm) and added to the autoclaved medium.

The cultures were incubated in dark at 24 ± 2 °C for 2 weeks and then at 24 ± 2 °C and 16-h photoperiod with irradiance of 40 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (*Philips*, Mumbai, India). Well-developed anther derived plantlets were removed from cultures and washed in sterilized distilled water to remove the traces of media. These plantlets were transplanted to plastic cups (7.5 × 8 cm) containing a mixture of autoclaved *Vermiculite*, sand and garden soil (1:1:1). The plants were grown in a plant growth chamber for 2 weeks under temperature 24 ± 2 °C, relative humidity 80 % and irradiance of 40 μmol m^{-2} s^{-1}, (16-h photoperiod) before transferring to greenhouse.

For embryo induction, 60 anthers were cultured in each treatment. For plantlet regeneration, mature embryos were cultured and all the experiments were repeated three times. The cultures were observed periodically and morphological changes were recorded at weekly intervals. The number of embryogenic anthers, embryos and plantlets produced in each treatment were counted and results expressed as percentage of embryogenic anthers, number of embryos and plantlets per treatment. The number of embryos was statistically analyzed by *ANOVA* and mean values were separated according to Duncan's multiple range test.

The anthers cultured on B5 medium supplemented

Table 1. Effect of different sugars on androgenesis of niger cv. Ootacamund. Mean values followed by same letters are not significantly different according to DMRT at P = 0.05. 60 anthers were cultured in each treatment. No embryos were developed on medium without sugars or at concentration 0.05 M. Also on medium with maltose at concentration higher than 0.25 M no embryos were observed.

Sugar	Conc. [M]	Responding anthers [%]	Number of embryos [treatment^{-1}]	Number of embryos [responding anther^{-1}]	Plantlet regeneration [%]
Sucrose	0.10	13.30	9.33k	1.50	44.44
	0.15	31.66	29.66c	1.56	58.42
	0.20	46.66	57.66a	2.05	68.78
	0.25	38.33	38.33b	1.66	54.78
	0.30	26.66	25.66d	1.60	45.45
	0.35	21.66	20.00e	1.53	31.66
	0.40	11.66	4.66mn	0.80	28.57
	0.45	8.33	2.33o	0.66	14.28
	0.50	3.33	1.33o	0.40	0.00
Glucose	0.10	11.6	0.00p	0.00	0.00
	0.15	28.33	6.33lmn	0.37	22.22
	0.20	36.66	11.00j	0.43	27.27
	0.25	33.33	13.00hi	0.65	25.64
	0.30	26.66	15.66fg	0.97	23.40
	0.35	23.33	18.66e	1.33	25.00
	0.40	21.66	19.66e	1.51	23.72
	0.45	18.33	13.66hi	1.24	21.95
	0.50	13.33	8.33kl	1.04	12.00
Fructose	0.10	8.33	0.00p	0.00	0.00
	0.15	20.00	0.00p	0.00	0.00
	0.20	33.33	5.33mn	0.26	18.75
	0.25	26.66	7.33klm	0.48	22.72
	0.30	21.66	13.00hi	1.25	10.25
	0.35	13.33	16.00f	1.83	6.25
	0.40	11.66	17.33f	2.07	3.80
	0.45	6.66	10.66j	2.06	3.12
	0.50	3.33	4.33mn	2.16	0.00
Maltose	0.10	8.33	5.66mn	1.13	17.64
	0.15	16.66	13.66hi	1.36	12.19
	0.20	11.66	11.33j	1.61	8.80
	0.25	6.66	8.33kl	2.16	4.00

with 10 µM 2,4-D and 2 µM KIN and various concentrations of sucrose, glucose, fructose and maltose grew in size and turned yellowish brown in two weeks of culture. Embryogenic callus development was observed in another two weeks (Fig. 1*A*). The globular embryos developed as protuberances (Fig. 1*B*) from the embryogenic calli in the next fortnight. There was significant difference among the type and concentration of sugars on embryo induction (Table 1).

Anthers did not respond on medium devoid of sugars and 0.05 M sucrose. Higher concentration of sucrose induced embryogenic callus and the percent of responding anthers as well as number of embryos per treatment increased from 0.15 to 0.25 M (Table 1). 46.6 % of anther produced an optimum of 57.66 embryos on medium containing 0.2 M sucrose in 6 weeks. Anthers cultured on medium containing 0.15 - 0.5 M glucose and 0.2 - 0.5 M fructose induced embryogenic callus. 33.33 % of anthers produced 13.0 embryos on medium supplemented with 0.25 M glucose. Maltose was not beneficial in embryo induction even though some concentrations (0.1 - 0.25 M) have induced embryogenic callus (Table 1). In the present study, sucrose was found superior for induction of embryos from anthers compared to glucose, fructose or maltose. Similarly, sucrose has also been a more suitable carbon source for induction of embryogenesis from cultured anthers in *Brassica oleracea* (Yang *et al*. 1992), *Cucumis sativus* (Ashok Kumar and Murthy 2004). Guo and Pulli (2000) reported that in *S. cereale,* the number of embryos/calli per dish was decreased from 116 to 90 as the concentration of sucrose increased from 0.25 to 0.50 M in the induction medium. The beneficial effect of maltose in inducing embryogenesis from anther cultures has been reported for barley (Finnie *et al*. 1989) and wheat (Karsai *et al*. 1994). In niger anther cultures, maltose showed poor response and only few concentrations (0.10 - 0.25 M) have induced embryogenesis in lesser percent of anthers (Table 1).

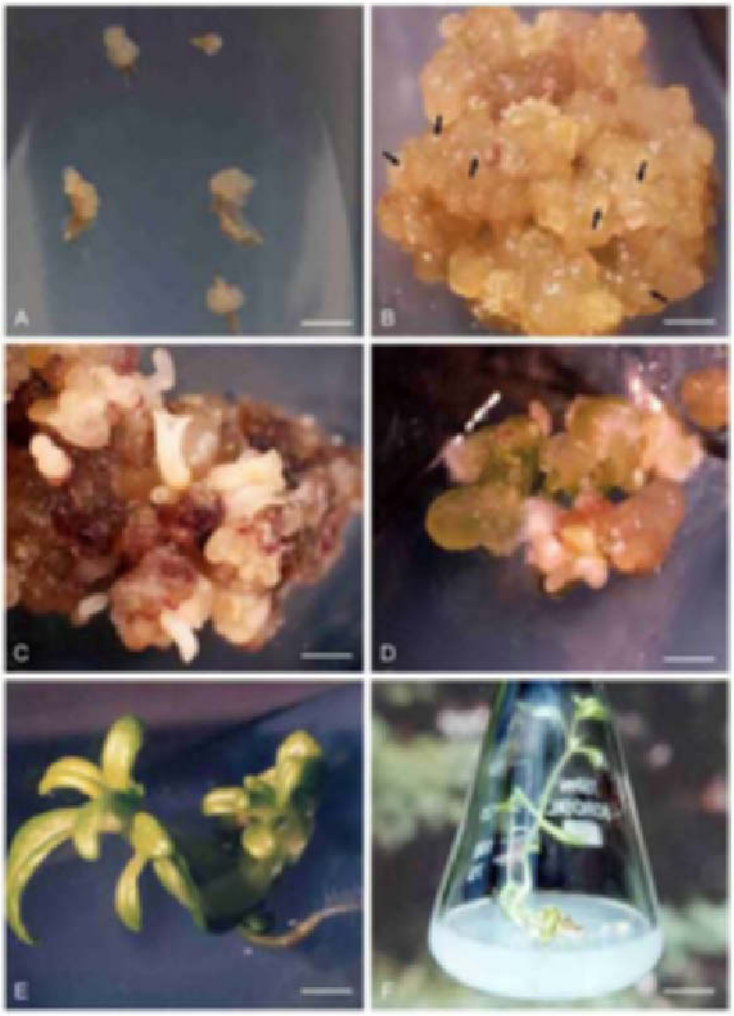

Fig. 1. Embryogenesis and plant regeneration in cultured anthers of niger cv. Ootacamund. *A* - Callus induction from anthers (*bar* = 0.5 mm). *B* - Callus showing the development of globular stage embryos (*bar* = 1.5 mm). *C* and *D* - Torpedo and cotyledonary stage embryos (*bar* = 2 mm). *E* - An anther derived plantlet (*bar* = 1.47 cm). *F* - Regenerated plantlet on embryo germination medium (*bar* = 1 cm).

Embryogenic calli containing globular embryos were subcultured to embryo differentiation medium (B5 medium supplemented with 0.09 M sucrose and 0.5 μM BA) upon which embryo differentiated into torpedo (Fig. 1*C*) and cotyledonary embryos in four weeks. Transferring of torpedo and cotyledonary embryos to maturation medium was essential in niger, otherwise embryo maturation was hindered. Torpedo and cotyledonary embryos in masses were transferred to embryo maturation medium (B5 medium supplemented with 0.09 M sucrose and 10 μM ABA) and upon which cotyledonary embryos turned green (Fig. 1*D*) and developed normally in another two weeks. Similar to the present results ABA treatment was found necessary for preventing precocious germination of embryos and normal development of embryos in *Cucumis sativus* (Ashok Kumar and Murthy 2004, Zhang *et al*. 2006). Mature embryos developed into plantlets on germination medium containing 0.09 M sucrose in another two weeks (Fig. 1*E*,*F*). Among the different sugars, sucrose favored conversion of plantlet regeneration with highest frequency (Table 1). Similarly, Metwelly *et al*. (1998) reported that, induction medium containing 0.44 M sucrose resulted in greatest frequency of plantlet regeneration from cultured anthers of C*ucurbita pepo.*

The plantlets were transplanted to potting mixture of *Vermiculite*, sand, and garden soil (1:1:1) 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.

In conclusion, embryo induction, differentiation, maturation and germination media have been worked out in the present studies from the anther culture of niger, and sucrose was found to be the most effective carbon source for embryogenesis and plantlet regeneration.

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