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In vitro plant regeneration from callus of niger (*Guizotia abyssinica* Cass.) cv. Sahyadri

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Abstract Callus formation was achieved with root, hypocotyl, and cotyledon explants of niger (*Guizotia abyssinica* Cass.) cultivar Sahyadri on Murashige and Skoog medium containing 0.5 mg l^{-1} β-indoleacetic acid + 1.5 mg l^{-1} 6-benzylaminopurine (BAP). Hypocotyl and cotyledon-derived calli when transferred onto a medium with 0.5 mg l^{-1} BAP produced an average of 12–32 shoots/callus culture. The callus retained its potential for shoot regeneration for more than 19 months. The shoots formed an extensive root system and were transferred to pots kept in a greenhouse, where the survival rate was 98%. The plantlets flowered in vitro if transfer to fresh medium or to soil was delayed by 40–50 days. All regenerants were diploid with 2n=30.

Key words *Guizotia abyssinica* · Prolonged callus culture · Plant regeneration

Abbreviations B_5 Gamborg's medium · *BAP* 6-Benzylamino purine · *2,4-D* 2,4-Dichlorophenoxyacetic acid · *IAA* β-Indoleacetic acid · *Kin* Kinetin · *LS* Linsmaier and Skoog's medium · *MS* Murashige and Skoog's medium · *NAA* α-Naphthaleneacetic acid · *WH* White's medium

Introduction

Niger (*Guizotia abyssinica* Cass., Asteraceae) is cultivated in India, Ethiopia, and other tropical regions for its oil-yielding seeds. Although this crop is one of the important sources of edible and industrial oil in the tropics, it has not received much attention for yield improvement. This situation has changed recently initiatives in both industrialized and developing countries.

To improve niger by genetic engineering, an essential prerequisite is the development of a procedure for efficient in vitro regeneration and transformation. Attempts in this direction have so far been restricted to either multiple shoot formation from shoot tips (Ahmad and Pande 1988), seedling explants (Nikam and Shitole 1993; Sarvesh et al. 1993a), or embryoid and shoot regeneration from anthers (Sarvesh et al. 1993b).

The goal here was to establish a reproducible in vitro regeneration system. We report on in vitro callus induction, high-frequency shoot regeneration, rooting, and successful hardening of *G. abyssinica*.

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Materials and methods

Plant material

Seeds of *G. abyssinica* Cass. cv. Sahyadri were surface cleaned by treatment with Teepol (Glaxo India) in distilled water for 2 min, sterilized in 0.1% $HgCl_2$ for 8 min, and rinsed in sterile water (5×10 min). The seeds were then transferred to sucrose (1%)-agar (0.8%) medium for germination. Explants were taken from the roots, hypocotyls, and cotyledons of 5- to 7-day-old seedlings.

Culture conditions

Entire cotyledons (18 mm^2), hypocotyl (8 mm) and root (8 mm) explants were transfered to 20 ml Murashige and Skoog (MS) (1962) medium (0.8% agar, 3.0% sucrose, pH 5.8) supplemented with kinetin (Kin), 6-benzylaminopurine (BAP), α-naphthaleneacetic acid (NAA), β-indoleacetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D) (0.1–5.0 mg l^{-1}) either individually or in combination, as well as to Linsmaier and Skoog (LS) (1965), White (WH) (1963) and Gamborg et al. (B_5) (1968) media. Once the optimum phytohormone concentration had been established for callus induction, calli were maintained on MS medium supplemented with 0.5 mg l^{-1} IAA + 1.5 mg l^{-1} BAP over 30 passages (30 days each). For each subculture 250±12 mg of fresh callus was transferred. Media were adjusted to pH 5.8 and autoclaved at 1.4 kg cm^{-2} and 121°C for 20 min. The cultures were maintained in 150×25 mm test tubes with non-

absorbent cotton plugs containing 20 ml medium at 25±2°C under a 9-h photoperiod with cool white fluorescent light (4–5 W m^{-2}). After 30 days of growth, calli were harvested, weighed, and dried in a hot air oven at 60°C until a constant weight was achieved (48 h).

Shoot regeneration

Callus was transferred in fragments of about 300 mg to MS, LS, WH and B_5 media without phytohormones as well as with IAA, NAA (0.1–1.0 mg l^{-1}), and Kin, BAP (0.1–3.0 mg l^{-1}) either singly or in combination.

Rooting of shoots

Separated shoots (1–2 cm long) were transferred to MS, LS, WH and B_5 media without phytohormones as well as with IAA or NAA (0.1–5.0 mg l^{-1}).

Chromosome counts

Root apices (1 cm) were excised from the regenerated plantlets to determine the chromosome number using the squash method (Jensen 1962). The tissues were pretreated with para-dichlorobenzene for 100 min at room temperature and fixed in a mixture of ethanol and glacial acetic acid (3:1) at 4°C for 24 h, hydrolysed in 1 N HCl at 60°C for 20 min, and stained with aceto-orcein.

Hardening

Rooted plants were rinsed free of agar and placed in pots containing sterilized soil. The plants were taken from the culture tubes and placed in the shade (maximum light 34.96 W m^{-2}, temperature 25±4°C, humidity 80–90%) and irrigated with tap water at 3-day intervals. For the first 3 weeks, the plants were covered with inverted transparent plastic cups to maintain high humidity levels; the cups were removed frequently in the last week. The plants were transferred to natural conditions (maximum light 550 W m^{-2}, temperature 28±7°C, humidity 50–90%) 2 weeks later.

All the experiments described here were repeated at least three times using a minimum of seven replicates/treatment.

Results and discussion

Callus formation

Callus formation of root, hypocotyl, and cotyledon explants was observed 1 week after the initiation of the culture. Addition of 0.5–2.00 mg l^{-1} phytohormone to the medium promoted callus induction in all three explants types. MS medium supplemented with 0.5 mg l^{-1} IAA and 1.5 mg l^{-1} BAP proved to be the most suitable for establishing callus culture. Root and cotyledon explants were superior to hypocotyl explants for callus induction.

Callus growth

Extensive callus growth occurred in medium with 0.5 mg l^{-1} IAA+1.5 mg l^{-1} BAP; good growth was also obtained with 0.25 mg l^{-1} NAA+1.5 mg l^{-1} BAP or Kin. Of the four media tested, MS was found to be superior to LS, WH, and B_5 (Table 1). With routine subculturing to fresh medium every 4 weeks, pale-green nodular calli developed which have been kept in an actively growing condition in the laboratory for more than 30 months. Similar results have been reported for other Asteraceae: *Lactuca sativa*, *Crepis capillar*, and *Helianthus annuus* (Flick et al. 1983).

Table 1 Effect of inorganic salts and vitamins of MS, LS, WH, and B_5 media with 0.5 mg l^{-1} IAA+1.5 mg l^{-1} BAP on callus growth in niger (*Guizotia abyssinica* Cass.) cv. Sahyadri. Results are the mean of seven replicates ±SD

Medium	Callus growth		
	Root callus dry weight (mg)	Hypocotyl callus dry weight (mg)	Cotyledon callus dry weight (mg)
MS	179 ± 1.10	148 ± 3.31	185 ± 3.74
LS	120 ± 4.23	140 ± 7.92	170 ± 4.58
WH	138 ± 2.16	131 ± 4.24	175 ± 0.17
B_5	157 ± 7.13	137 ± 2.00	179 ± 7.32

Organogenesis

Attempts to induce shoot regeneration in the callus of root explants using MS, LS, WH and B_5 medium supplemented with various auxin and cytokinin combinations in varying concentrations were unsuccessful. However, calli obtained from hypocotyl and cotyledon explants cultured on media supplemented with IAA (0.5–1.0 mg l^{-1}) + BAP (1.0–1.5 mg l^{-1}) or NAA (0.25 mg l^{-1}) + BAP (1.0–1.5 mg l^{-1}) or BAP (1.5–2.0 mg l^{-1}) alone responded with shoot regeneration (Table 2). Addition of BAP at 0.25 mg l^{-1} to the medium induced shoot regeneration within 2 weeks of callus culture. Usually, 4–17 shoots developed per culture (Table 2). Raising the level of BAP to 0.5 mg l^{-1} optimized the number of shoots (12–32 per culture, Table 2). Of the four media tested, MS was found to be superior to LS, WH, and B_5. Sarvesh et al. (1993b) achieved 8–12 shoots per culture regenerated from anther callus. Addition of NAA or IAA (0.1–0.25 mg l^{-1}) in shoot regeneration medium was found to inhibit regeneration. Callus obtained on 2,4-D-containing media did not respond with organogenesis.

For several members of the Asteraceae, Kin was found to be the most effective hormone in regeneration media (Flick et al. 1983). However, niger callus incubated on media containing 0.1–3.0 mg l^{-1} Kin did not regenerate shoots. Similar results were obtained with *H. annuus* (Paterson and Everett 1985) and *Carthamus tinctorius* (Orlikowska and Dyer 1993)

Regeneration from 3- to 4-year-old callus of *C. capillar* (Hüsemann and Reinert 1976) and 14-month-old callus of *Brachycome dichromosomatica* (Gould 1979) has been reported. In niger, shoot regeneration potential in the callus derived from hypocotyl and cotyledon persisted for over 19 months.

Table 2 Shoot regeneration in callus of niger (*G. abyssinica* Cass.) on MS medium supplemented with various concentrations of BAP. Results are the mean of seven replicates (15×3) ±SD (*HIB* Hypocotyl callus on MS+0.5 mg l^{-1} IAA+1.5 mg l^{-1} BAP, *HNB* hypocotyl callus on MS+0.25 mg l^{-1} NAA+1.5 mg l^{-1} BAP, *HB* Hypocotyl callus on MS+1.5 mg l^{-1} BAP, *CIB* cotyledon callus on MS+0.5 mg l^{-1} IAA+1.5 mg l^{-1} BAP, *CNB* cotyledon callus on MS+0.25 mg l^{-1} NAA+1.5 mg l^{-1} BAP, *CB* cotyledon callus on MS+1.5 mg l^{-1} BAP, * no shoot regeneration observed)

BAP (mg l^{-1})	Number of shoots per culture					
	HIB	HNB	HB	CIB	CNB	CB
0.00	*	*	*	*	*	*
0.1	*	*	*	*	*	*
0.25	4.8 ± 1.9	6.1 ± 2.1	14.7 ± 4.5	5.7 ± 2.4	4.8 ± 2.5	16.5 ± 4.1
0.5	20.7 ± 8.8	21.2 ± 5.2	24.8 ± 5.7	23.1 ± 6.0	21.8 ± 5.8	26.7 ± 5.1
1.0	15.5 ± 5.4	11.0 ± 3.0	7.0 ± 4.3	13.7 ± 4.1	7.2 ± 2.5	10.8 ± 2.6
1.5	9.1 ± 2.0	6.0 ± 2.2	2.14 ± 0.9	9.8 ± 2.2	4.2 ± 1.4	4.0 ± 1.5
2.0	*	*	*	*	*	*
3.0	*	*	*	*	*	*

Fig. 1A, B *Guizotia abyssinica* Cass. Sahyadri **A** callus mediated rooted shoot flowering in vitro on MS medium with 1.0 mg l^{-1} IAA (*bar* 5 mm). **B** Potted plant under natural conditions (*bar* 10 mm)

Root induction and growth

Root formation on shoots was induced by transplanting excised shoots to hormone-free MS, LS, B_5, or WH media for 12 days. No significant difference in the number of shoots with roots was found on media with varying concentrations of IAA and NAA (Table 3). The best root growth in all shoots in the presence of 0.5 mg l^{-1} NAA. Higher concentrations of IAA (1.0–3.0 mg l^{-1}) and NAA (1–2 mg l^{-1}) induced callus at the base of the shoots. When transfer of the rooted shoots to fresh media or soil was delayed by 40–50 days, additional adventitious roots were formed, and all the shoots flowered in vitro (Fig. 1A). Thus rooting in niger may be achieved more easily than in other Asteraceae, e.g., *Gerbera* (Pierik et al. 1975) and *Chrysanthemum* (Earle and Langhans 1974). Early flowering in vitro would be valuable for analyzing genetic and physiological factors controlling the induction of capitula and for breeding plants with improved characteristics.

Hardening and characterization of regenerants

Rooted plants were transferred to pots and hardened, as described in Materials and methods, with a 2% loss during acclimatization. After 1 month of growth, these plants ap-

Table 3 Effect of MS medium containing different levels of IAA and NAA on the initiation of roots in *G. abyssinica* Cass. shoots. Results are the range followed by the means of seven replicates (28×3) ±SD

Auxin (mg l^{-1})	Number of days required for induction of roots			
	IAA		NAA	
0.00	12–17	15.0 ± 1.33	12–17	15.0 ± 1.33
0.1	7–12	9.9 ± 1.66	7–10	9.1 ± 0.78
0.2	7–10	8.7 ± 1.16	5–7	6.2 ± 0.83
0.5	6–9	6.9 ± 0.99	3–5	3.8 ± 2.12
1.0	13–16	14.3 ± 1.34[a]	6–10	8.0 ± 1.32[a]
2.0	13–16	14.6 ± 1.07[a]	8–10	9.4 ± 0.61[a]
3.0	14–18	16.3 ± 1.70[a]		[b]

[a] Callus-mediated rooting
[b] Callusing with hyperhydricity

peared quite similar to those in their natural environment. All regenerants had a chromosome number of 2n=30. Plantlets grew to a height of 40–74 cm, flowered (Fig. 1B), and completed their life cycle within 70–90 days.

These results demonstrate an efficient protocol for the in vitro propagation of niger.

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