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Anther Culture of Niger

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Introduction

Haploids derived from anther and microspore cultures have considerable potential in plant breeding because of time saved by the reduction of classical selection cycle period and the genetic value of isogeneic lines or homozygous dihapliods. Also, the presence of a single set of chromosomes allows the detection of mutations controlled by recessive genes and recovery of unique recombinants.

Guizotia abyssinica (L.f.) Cass. commonly known as niger, belongs to the family Asteraceae and is an oilseed crop cultivated in Indian Subcontinent and East African countries. Seeds contain about 30-50% of a yellow, edible, semi-drying oil with little odor and pleasant nut taste which is valuable dietetically (Anonymous 1956; Weiss 1982). The crop by virtue of the enormous variability it presents for various characters of economic importance offers tremendous scope for improvement through heterosis breeding. However, self-incompatibility mechanism causes serious difficulty for inbred line development and maintenance (Getinet and Sharma 1996). Alternative method for homozygous line production is by induction of haploids by anther or micorspore culture and subsequent production of double haploids. Anther culture studies has been initiated by Survesh et al. (1993) and have reported callus mediated regeneration in niger. We are involved in anther culture studies and procedures for reliable haploid production in niger is outlined here.

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Procedure

Growth of donor plants

The physiological conditions of the donor plants will significantly influence anther culture success. Green house grown plants and field grown plants can be used successfully. However, plants grown in controlled conditions in pots with soil-sand-farmyard manure (1:1:1) in growth chambers are yielded better results. A 28-15℃ day-night cycle is satisfactory and 16-hrs photoperiod is generally employed. The light source of 450-500 μmole m^{-2} s^{-1} is excellent for healthy development of plants and flower bud induction.

Bud selection and anther staging

In niger, as in many other species, the uninucleate micorospore stage is the most responsive in culture. The pollen development stage was determined when capitulum began to swell. Pollen stage and development status were observed and recorded. The size of capitula can be correlated with the stage of microspores inside the anther. The young and immature capitula of 5-6 mm in diameter were having uninucleated microspores and these buds are conveniently used for anther culture. The sizes of the capitulum corresponding to stage and embryo yield are presented in table 1. The results showed uninucleate stage is most suitable and yields optimal number of embryos. The color of anther can also be correlated with the microspore developmental stages. The anthers are initially green in color when they are young, turns whitish-green, subsequently they turned yellow and brown at maturity. The anthers,

Table 1. Effect of capitulm (bud) size and stage of anther on androgenic embryo yield of niger genotype UNS-4[b].

Capitulum size	Microspore stage	No.of embryos/anther[a] (Mean ± S E)
3-5 mm	uninucleated with central nucleus	7.5±1.5
5-6 mm	uninucleated with lateral nucleus	10.2±2.1
6-8 mm		Nil

[a] Average number of embryos per 100 cultured anthers.
[b] Anthers are cultured on B5 medium supplemented with 2,4-D (2 µM) + BA (1µM) with 4% sucrose.

which are whitish green, contained uninucleate staged micorspores. Accurate staging can be achieved by fixing capitula in Carnoy's (6 parts of ethanol: 3 parts of acetic acid: 1 part of chloroform) solution and staining anthers in aceto-carmine by smear method. The most suitable material for anther culture is the selection of main capitula (buds), which are emerging from flowering branches. However, the axillary capitula may be also used if the donor material is valuable.

Bud pretreatment

Pretreatment of capitula at 4°C for one day prior to anther culture is reported to enhance embryognenic callus induction in niger (Survesh et al. 1993). However, in our experiments pretreatment of capitula at 4-6°C for 5 days prior to anther culture, have responded better to the cultural regimes and developed embryos.

Surface sterilization and anther extraction

Excised capitula were surface sterilized by a 15 minutes immersion initially in 10% (v/v) solution of sodium hypochlorite and subsequently in 0.1% mercuric chloride (w/v) for 5 minutes. Then they were washed in sterile distilled water. Flower buds were removed from the capitula and placed on the stage of dissection microscope, which has been wiped with 70% ethanol. Carefully individual anthers were separated and care must be taken not to puncture or crush the anther locule, as damaged anthers are rarely repond to *in vitro*. The anthers should be placed horizontally on the medium so that the anther flat side is in contact with the medium.

Culture medium

Gamborg et al. (1968) medium (B5 medium) was used for niger anther culture. The medium was supplemented with 2,4-D alone at 0.5, 1.0, 2.0 and 5.0 µM concentrations and combination of 2,4-D (1.0, 2.0 and 5.0 µM) and BAP (0.5, 1.0, and 2.0 µM) were used. Various levels of sucrose (2%, 4%, 6%, and 8%) were used. The media was adjusted to 5.8 and was solidified with 0.8% agar (Qualignes, Mumbai, Inaida). The media was autoclaved at 121°C at 104 K Pa pressure for 20 minutes.

Both solid and liquid media were used for anther culture. When solid medium was used, 20 ml of medium was taken in 85×20 mm petri dishes (Himedia, Mumbai, India) and 20×150 mm test tubes (Borosil, Mumbai, India). When liquid medium was used, 20 ml of medium was taken in 85×20 mm petri dishes, and cultures were maintained on rotary shaker with speed of 100 revolutions per minute.

Culture conditions

The cultures were incubated in dark for 10 days at 45°C. After that, the cultures were incubated under cool white fluorescent light with 16-h photoperiod (40 µmoles m^{-2} s^{-1}) and 60-65% relative humidity.

Plant regeneration

Plantlets were not developed from embryos in the anther culture medium, and an embryo culture procedure is required. The embryo culture medium consists of B5 salts and vitamins with 2% sucrose without growth regulators. It is essential to culture the embryos soon after emergence, as prolonged maintenance on the anther culture medium will lead to senescence. Embryo culture conditions include maintenance of 10 embryos per 85×20 mm petri dishes (20 ml of medium) at 25°C under white fluorescent light (40 µmoles $m^{-2}s^{-1}$, 16 h per day).

The frequency of embryo survival is dependent on the developmental stage. Most globular embryos do not survive, whereas the majority of bipolar embryos survive and turn green.

Table 2. Effect of various concentrations and combinations of growth regulators and sucrose on induction of embryos from anther of niger.[a]

Medium	Sucrose(%)	Growth regulator 2,4-D (μM)	BA (μM)	No. of anthers scored	No. of anthers responded*	No. of embryos/ anther*
B5	2	1.0	0.5	120	0	0
		2.0	0.5	120	0	0
		5.0	0.5	120	0	0
		1.0	1.0	120	0	0
		2.0	1.0	120	0	0
		5.0	1.0	120	0	0
		1.0	2.0	120	0	0
		2.0	2.0	120	0	0
		5.0	2.0	120	0	0
B5	4	1.0	0.5	120	14d	4.75cd
		2.0	0.5	120	17d	4.66cd
		5.0	0.5	120	13d	3.25d
		1.0	1.0	120	19d	5.65bc
		2.0	1.0	120	14ad	9.44ab
		5.0	1.0	120	21d	4.60c
		1.0	2.0	120	10d	2.74d
		2.0	2.0	120	23ad	4.95c
		5.0	2.0	120	47ad	7.27b
B5	6	1.0	0.5	120	15d	6.88b
		2.0	0.5	120	20d	4.95c
		5.0	0.5	120	17d	2.88d
		1.0	1.0	120	23cd	8.78ab
		2.0	1.0	120	55a	11.54a
		5.0	1.0	120	23cd	6.73b
		1.0	2.0	120	07d	3.00d
		2.0	2.0	120	33bc	4.75c
		5.0	2.0	120	50a	7.46b
B5	8	1.0	0.5	120	17d	3.70c
		2.0	0.5	120	12d	2.83d
		5.0	0.5	120	08e	2.37d
		1.0	1.0	120	09e	3.33d
		2.0	1.0	120	27c	7.0 b
		5.0	1.0	120	10e	2.85d
		1.0	2.0	120	05e	2.22d
		2.0	2.0	120	15d	3.46d
		5.0	2.0	120	21d	4.90cd

[a] Observations are made 6 weeks of culture.
* Mean values within column followed by different letter differ significantly according to Duncan's multiple range analysis at P= 0.05.

Transplantation of in vitro derived plants

The plantlets derived in vitro were initially transferred to plastic pots containing vermiculite and maintained them in growth chamber with high humidity and under a day/night temperature of 25/15℃ and 16 h photoperiod of 200 μmole m^{-2} s^{-1}. After two weeks the plants were transferred to green house where the humidity was 50%, day and night temperature were 28/15℃ and light intensity was 500 μmoles m^{-2} s^{-1} for

16 hr per day.

Cytological characterization of regenerants

For chromosome analysis of the anther derived plants, the root tips were pretreated with 0.05% colchicine for 2 h, fixation was done by using acetic-alcohol (1 part of acetic acid and 3 parts of absolute alcohol) and roots were squashed in orcein stain.

Results and Conclusions

Bud (capitulum) size and anther staging

One of the most critical requirements for successful induction of androgenesis *in vitro* is the stage of microspores at the time of anther excision and culture. In many species maximum response is obtained when anthers excised just before, during, or immediately following the mitosis of the microspore nucleus (Vasil 1980). Buds (capitula) of various sizes were tested for embryo induction. The bud size and color of the anther has been correlated with the developmental stage of anther stage. The capitula of size 5-6 mm in diameter possessed uninucleate-staged anthers and these buds conveniently used for the anther culture (Table 1). The anthers are initially green in color, turns whitish green and subsequently yellow and brown at maturity. The anthers, that are whitish green contained uninucleated microspores and upon culture of such anthers, they have yielded optimal results. Similarly, uninucleated anthers responded well to the cultural regimes in *B. oleracea* (Phippen and Ockendon 1990; Yang et al. 1992).

Bud pretreatment

Pretreatment of excised flower buds can help to improve anther response in culture. Survesh et al. (1993) used buds, which are chilled at 4°C for 24 hours for anther culture studies and induced embryogenic callus from anthers. In our studies buds were pretreated at 4-6°C for 5 days showed embryo induction on medium with specific combination of growth regulators and sucrose levels.

Induction of embryogenesis

Initial experiments were conducted by using genotype UNS-4. The results of effect of 2,4-D and BA combinations and various concentrations of sucrose on anther culture are summarized in table 2. Anthers cultured on medium containing 2,4-D alone (0.5 and 1.0 µM) and different levels of sucrose (2, 4, 6 and 8%) did not show any response. Anthers cultured on medium with higher concentrations of 2,4-D (2.0 and 5.0 µM) with different levels of sucrose developed only callus (data is not shown here). Similarly, anthers cultured on medium containing combination of 2,4-D (1.0,2.0 and 5.0 µM) and BA (0.5, 1.0 and 2.0 µM) with 2% sucrose also developed callus. However, the anthers cultured on medium containing 2, 4-D (1.0-5.0 µM) and BA (0.5-2.0 µM) with higher levels of sucrose (4-8%) have developed embryos directly on the surface of the anther. Anthers after one week of culture became brown and subsequently black brown and by fourth week developed whitish globular structures. Globular embryos first appeared as protuberances on the surface of the explants. Individual embryos, which enlarge into bipolar structures passed through typical globular, heart shaped, and cotyledonary stages. Among the 2,4-D and BA combination tested with various levels of sucrose (4-8%), optimum response and embryo induction was noticed on medium supplemented with 2 µM 2,4-D and 1 µM BA with 6% sucrose. Although embryos were visible on the surface within 4 weeks of culture, their differentiation to cotyledonary stage took another 2 weeks and embryo maturation was also on the same medium.

In earlier studies on niger anther culture, Survesh et al. (1993) have reported induction of embryogenic and non embryogenic callus. Anthers cultured on LS medium supplemented with 2 mg l^{-1} 2,4-D and 0.3 mg l^{-1} Kinetin have induced embryogenic callus and the anthers cultured on LS medium supplemented with 1mg l^{-1} BAP and 0.2 mg l^{-1} Kinetin produced non-embryogenic callus. In the present studies, direct embryo induction was achieved from anthers on B5 medium supplemented with 2,4-D and BA combination with high levels of sucrose. These results reveal that 2,4-D is the essential component in the medium for induction of embryogenesis. Similarly, there are various recent reports wherein the embryogenesis is induced from anthers, either in presence and 2.4-D alone or

under the influence of both auxin and cytokinin (Thengane et al. 1994; Faure et al. 1996; Marciniak et al. 1998; Metwally at al. 1998; Kiviharju and Tauriainen 1999).

Different concentrations of sucrose was tested during the anther culture and anthers cultured on B5 medium with 2,4-D and BA with 2% sucrose developed only callus. Whereas, anthers cultured on medium with 2,4-D and BA with higher level of sucrose (4-8%) have induced embryos without intervening callus. This indicates that higher level of sucrose along with growth regulators is essential for direct embryo induction from anthers of niger. Similarly, higher concentrations of sucrose have influenced the embryogenesis during anther/microspore culture of maize (Pescitelli et al. 1990), *Brassica campestries* (Ballie et al. 1992), *Cucurbita pepo* (Metwally et al. 1998), and *Linum usitatissimum* (Chen et al. 1998).

Genotypic variability

In another experiment, anthers of various genotypes were cultured on MS medium with 2,4-D (2 μM) and BA (1 μM). Embryo induction varies with different genotypes. Among the 12 genotypes tested, No. 71, JNC-1, NPJ-41, UNS-4 and Ootacamund genotypes responded well and more than 25% of anthers produced embryos.

Table 3. Genotypic variation on induction of embryos during the anther culture of niger.[a]

Genotype	No. of anthers cultured	Percentage of anthers which developed embryos (with S E)
No. 71	120	27±3
IET-71	120	16±1.0
IET-19	120	19±1.8
RCR-18	120	09±2.1
IGP-76	120	10±1.5
GA1	120	14±2.6
RCR-140	120	16±1.8
RCR-317	120	15±0.9
JNC-I	120	25±2.1
NPJ-41	120	28±3.4
UNS-4	120	55±4.1
Ootacamund	120	40±3.2

[a] Anther were cultured on B5 medium containing 2,4-D (2 μM) + BA (1.0 μM) with 6% sucrose.

Table 4. Comparison of solid and liquid media on embryo induction during anther culture of niger genotype UNS-4.[a,b]

Medium types	No. of embryos per anther (Mean S E)
Solid	11.1±2.6
Liquid	Nil

[a] Average number of embryos per 100 anthers.
[b] Anthers are cultured on B5 medium supplemented with 2,4-D (2 μM) + BA (1 μM) + 6% sucrose.

Whereas the frequency of response with other genotypes was low (Table 3). In many plants, it is observed that, the ability of anthers/microspores to form embryoids or callus *in vitro*, is under genetic control and is affected by the genotypic differences among the donors (Ockendon 1985; Faller and Turton 1990; Lazar et al. 1990; Baellie et al. 1992; Burnett et al. 1992; Yang et al. 1992).

Cultural conditions

Anthers cultured on liquid medium did not respond at all. However, on solid medium optimal of 11 embryos produced per anther (Table 4). Liquid nutrient media have been shown to be superior to agar media in *Brassica oleracea var. botrytis* (Yang et al. 1992). On the contrary, Phippen and Ockendon (1990) have reported influence of solid medium on embryo induction in the same species.

Regeneration of plantlets, transplantation and cytological characterization

Matured embryos were subcultured to half strength B5 medium containing 2% sucrose where they germinated into plantlets in 3 weeks. 47% of the embryos converted into plantlets. The regenerated plants were transferred to community pots successfully and percentage of survival was 65%. The haploid nature of the plantlets were confirmed by chromosome analysis and 90% of the plantlets were haploids with n=15 chromosomes and remaining were diploids.

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