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In vitro Regeneration of Four Ethiopian Varieties of Sesame (*Sesamum indicum* L.) using Anther Culture

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Abstract: The study aimed at developing a suitable and reproducible protocol of *in vitro* regeneration of haploid sesame (*Sesamum indicum* L.) plantlets using anther culture. Anthers of four Ethiopian varieties, namely: Hirhir, *Humera-1*, *Setit-1* and Non-Shatter were cultured *in vitro* to study their regenerating ability. Murashige and Skoog (MS) media supplemented with different concentrations and combinations of plant growth regulators (PGRs) were used. The highest callusing (56.20%) and callus weight (8.33 g) were observed in Hirhir cultured in MS medium supplemented with 2,4-D (2,4 dichlorophenoxyacetic acid) at 2.0 mg L^{-1} + BAP (benzylaminopurine) at 1.0 mg L^{-1}. Shooting response was studied using green/friable calli of the varieties cultured in MS medium supplemented with BAP at the concentrations of 0.5, 1.0, 1.5 and 2.0 mg L^{-1} + NAA (naphthalene acetic acid) at 1.0 mg L^{-1}. Calli of Hirhir cultured in MS medium supplemented with BAP at 2.0 mg L^{-1} + NAA at 1.0 mg L^{-1} were the best in terms of percentage shooting and number of days to shooting. Non-Shatter showed the weakest response to the same treatment. Rooting of shoots was studied with 0, 0.25, 0.5, 0.75 and 1.0 mg L^{-1} of IBA + 0.5 mg L^{-1} of NAA. In this case too, whereas Hirhir cultured in MS medium supplemented with 0.25 mg L^{-1} IBA (indole-3-butyric acid) +0.5 mg L^{-1} NAA showed the best response –87.15% in mean rooting, 11.66 mm in mean root length and 17.80 in mean days to rooting; Non-Shatter cultured in the same medium yield weakest response. Acclimatization responses of rooted *in vitro* seedlings were studied in coco peat and soil medium (comprising sand, cow dung and garden soil at a ratio of 2:1:1). Seedlings of Hirhir planted in both media gave better survival rate than that of the other varieties. The survival rates of Hirhir planted in coco peat and soil media were 66.7 and 50.0%, respectively.

Key words: Acclimatization, callusing, rooting, shooting

INTRODUCTION

Sesame (*Sesamum indicum* L.) known as the queen of oil seeds belongs to family Pedaliaceae. The family comprises about 40 species, many of which being wild. *S. indicum* L. is the only cultivated species (Subramanian, 2003), covering a total area of over 7,700,276 hectares and annual production of 3,976,968 tons (FAOSTAT, 2012). Sesame oil is used domestically for cooking and medicinal purposes (Wijnands *et al*., 2007). Sesame seed contains 50-60% oil, 25% protein and some antioxidants such as sesamolin and sesamin used as active ingredients in antiseptics, bactericides, viricides, disinfectants, moth repellants, and anti-tubercular agents (Bedigian *et al*., 1985). It is also a good source of calcium, tryptophan, methionine and many minerals (Johnson and Emino 1979).

Anther culture techniques are used in producing haploid and doubled haploid cells. The haploid cells, in turn, are used in producing cell lines with complete homozygosity. The homozygous cell lines are used in: genetic engineering research, producing new varieties and hybrids, selecting stable and resistant cell lines against biotic and abiotic stresses and in purifying male cell lines (Galli *et al*., 1998). Anther culture has recently attracted considerable interest in supplementing efforts of rapid production of haploid and inbred lines, thus hybrid cultivars (Jain *et al*., 1996; Keller and Armstrong, 1978; Wenzel *et al*., 1977). Haploid lines generated through anther culture techniques are used in producing homozygous diploid varieties or parental lines with added genetic divergence. Also, doubled haploids give rise to homozygosity of the descendants, a time saving

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procedures in developing new varieties (Kasha *et al*., 1990). Hence, anther culture and subsequent plant regeneration techniques offer efficient alternative to conventional breeding methods. The article reports results of a study aimed at developing suitable and reproducible protocol of *in vitro* regeneration of four Ethiopian sesame varieties, called Hirhir, Humera-1, Setit-1 and Non-Shatter, using anther culture technique.

MATERIALS AND METHODS

The study is carried out at Mekelle Agricultural Research Center, northeastern Mekelle, Ethiopia between January 2012 and February 2013. Explant donor plants were obtained from the Center. Donor plants were, then, planted in potted soil collected from the backyard of the Research Center. Pots were placed in greenhouse of the Center and were regularly watered with tap water.

Media used for anther culture: MS media (Murashige and Skoog, 1962) supplemented with 0.5, 1.0, 1.5 and 2.0 mg L^{-1} 2,4-D plus constant concentration of BAP at 1.0 mg L^{-1} were used in callusing. MS media supplemented with 0.5, 1.0, 1.5 and 2.0 mg L^{-1} BAP plus constant concentration of NAA at 1.0 mg L^{-1} were used in shooting, while half strength MS media supplemented with 0.25, 0.5, 0.75 and 1.0 mg L^{-1} IBA plus constant concentration of NAA at 0.5 mg L^{-1} were used in rooting. All treatments were compared against each other and the treatments with no PGRs, regarded as controls.

Anther collection and inoculation for callusing: Flower buds of the four varieties were collected in the morning before 10 am. Anthers were carefully cut with forceps and trimmed with scissors prior to disinfection and detachment from flower buds. Surface sterilization of buds was done by gently washing them with running tap water three times; then in 70% alcohol for 45 sec followed by 5% KCl for 10 min and 0.1% $HgCl_2$ for 1 min. Buds were rinsed three times with sterilized distilled water before excision of intact anthers. Then, the buds were wrapped in aluminum foil and kept in a refrigerator at 5°C for 24 h. Finally, anthers were aseptically cut from flower buds using fine tweezers and inoculated into 8 cm Petri dishes, each containing 10 mL MS medium supplemented with different concentrations and combinations of PGRs.

Calli collection and inoculation for shooting: Regenerated calli attained convenient size five and half to seven weeks after inoculation of anthers. Then, the calli were aseptically taken from the regeneration media and transferred to sterile culture vessels inside laminar air flow cabinet. Finally, they were cut into few pieces and placed in small vials containing shooting media.

Collection of shoots and inoculation for rooting: Regenerated shoots were aseptically inoculated onto rooting media containing MS base. Side by side, sub-cultured calli were let to proliferate and differentiate into shoots. Shoots of 5 to 7 cm long were rescued aseptically from the cultured vials; were separated from each other; and cultured in vials with freshly prepared rooting media. Vials containing plantlets were incubated under continuous light.

Transplanting rooted shoots for acclimatization: Plantlets with well-developed roots were subsequently taken out of culture vials. The agar was carefully washed off of the plantlets by rinsing with tap water. Plantlets were, then, planted in coco peat filled in polystyrene trays and soil medium (with sand, cow dang and garden soil at ratio of 2:1:1) filled in plastic pots. The trays and pots were transferred to growth chamber and placed in well-controlled, high-tech greenhouse environment for *Ex vitro* acclimatization. Plantlets were covered with plastic sheets and kept in 70% shade netted, high relative humidity greenhouse at 25°C for 10 to 15 days.

RESULTS AND DISCUSSION

Callus induction: The study showed that callusing response of anthers of the varieties-measured by days to callusing, percentage of callusing, and weight of callus-varies with varying concentrations of 2,4-D of the MS callusing media (Table 1). Callusing media with no supplements of PGRs did not cause any callusing. Explants of Hirhir cultured on MS media supplemented with 2,4-D at 2.0 mg L^{-1} + BAP at 1.0 mg L^{-1} have the shortest callusing time; i.e., 34.00 days. On the other hand, explants of Non-Shatter cultured on MS media supplemented with 2,4-D at 0.5 mg L^{-1} + BAP at 1.0 mg L^{-1} and 2,4-D at 1.0 mg L^{-1} + BAP at 1.0 mg L^{-1} took significantly longest time to callus than Hirhir; i.e., 54.00 and 51.66 days, respectively ($p \leq 0.05$). When the responses of all the varities are examined, delayed callusing is observed in MS media supplemented with lower concentration of 2,4-D (i.e., 0.5 m L^{-1}). Similar callusing responses were reported in *Brassica* species where fast callusing responses were observed with MS media supplemented with 0.5 mg L^{-1} 2,4-D and slow callusing were observed with MS media supplemented with 0.1 mg L^{-1} 2,4-D (Sayem *et al*., 2010).

As callusing responses of the varieties are examined in terms of percentage callusing, the response of Hirhir

Table 1: Interaction varying concentration of 2,4-D + 1.0 mg L^{-1} BAP and varieties of *S. indicum* L. on callusing

	Treatment (mg L^{-1})		Mean response		
Varieties	2,4-D	BAP	Days to callusing	Percent callusing	Weight of callus
Hirhir	0.5	1.00	47.33^{cd}	37.50^{g}	1.67^{g}
	1.0	1.00	41.00^{fg}	45.70^{e}	6.67^{bc}
	1.5	1.00	38.33^{g}	50.00^{b}	7.33^{a}
	2.0	1.00	34.00^{h}	56.20^{a}	8.33^{a}
Humera-1	0.5	1.00	49.33^{bcd}	29.10^{j}	1.33^{g}
	1.0	1.00	48.67^{bcd}	31.20^{i}	4.67^{e}
	1.5	1.00	45.67^{de}	38.17^{fg}	6.00^{cd}
	2.0	1.00	42.67^{ef}	41.60^{e}	6.67^{bc}
Setit-1	0.5	1.00	50.67^{abc}	31.67^{i}	1.33^{g}
	1.0	1.00	49.33^{bcd}	35.27_{h}	5.33^{ed}
	1.5	1.00	48.00^{bcd}	39.50^{f}	6.33^{c}
	2.0	1.00	45.67^{de}	43.70^{d}	7.33^{b}
Non shatter	0.5	1.00	54.00^{a}	18.70^{l}	1.33^{g}
	1.0	1.00	51.66^{ab}	22.10^{k}	3.67^{f}
	1.5	1.00	49.00^{bcd}	31.20^{i}	6.67^{bc}
	2.0	1.00	47.33^{cd}	32.60^{i}	7.33^{b}
*LSD		4.15	01.67	02.02	
**CV (%)		6.99	03.50	13.56	

Means in the same column with different letters are significantly different at $p \leq 0.05$. *LSD = Least significant difference; **CV = Coefficient of variation

cultured on MS media supplemented with 2,4-D at 2.0 mg L^{-1} + BAP at 1.0 mg L^{-1} was significantly greater than all other treatments (56.2%, $p \leq 0.5$). As comparisons were made within each variety, MS media supplemented with 2,4-D at 2.0 mg L^{-1} + BAP at 1.0 mg L^{-1} resulted in significantly higher callusing rate at 56.2, 41.6 and 43.7% for Hirhir, Humera-1, and Setit-1, respectively. Though comparably weak, Non-Shatter yielded higher callusing rate at higher concentrations of 2,4-D. Other authors (Badigannavar and Kururvinashetti, 1998; Punia and Bohorova, 1992) observed similar callusing responses with 2.0 mg L^{-1} 2,4-D. The weakest responder being Non-Shatter, all varieties had lowest callusing responses with MS media supplemented with 2,4-D at 0.5 mg L^{-1} + BAP at 1.0 mg L^{-1} -37.5, 29.1, 31.7 and 18.7% for Hirhir, Humera-1, Setit-1, and Non-Shatter, respectively ($p \leq 0.05$). Observations in which lower concentrations of 2,4-D resulted in lower callusing rate were reported in *Brassica* species (Alam *et al*., 2009; Sayem *et al*., 2010).

Analyses of callus weight showed that whereas higher concentrations of 2,4-D resulted in large callus mass, lower concentrations resulted in smaller callus mass in all varieties tested in the study. MS media supplemented with 2.0 mg L^{-1} of 2,4-D + 1.0 mg L^{-1} BAP yielded 8.33, 6.67, 7.33 and 7.33 grams of callus mass in Hirhir, Humera-1, Setit-1 and Non-Shatter, respectively. Callus mass of Hirhir is significantly greater than that of the other varities. MS media supplemented with 0.5 mg L^{-1} of 2,4-D + 1.0 mg L^{-1} BAP yielded 1.67, 1.33, 1.33 and 1.33 g of callus mass in Hirhir, Humera-1, Setit-1 and Non-Shatter, respectively.

Table 2: Interaction of varying concentration of BAP + 1.0 mg L^{-1} NAA and varieties of *S. indicum* L. on shooting

	Treatment (mg L^{-1})		Mean response	
Varieties	BAP	NAA	Days to callusing	Shooting rate (%)
Hirhir	0.0	0.00	34.33^{abc}	1.37^{h}
	0.5	1.00	30.00^{cd}	6.91^{ef}
	1.0	1.00	23.00^{fgh}	12.50^{cd}
	1.5	1.00	20.00^{ghi}	16.66^{b}
	2.0	1.00	16.00^{i}	22.20^{a}
Humera-1	0.0	0.00	32.00^{bcd}	2.08^{h}
	0.5	1.00	34.67^{abc}	2.75^{gh}
	1.0	1.00	30.33^{bcd}	5.52^{fg}
	1.5	1.00	24.33^{efg}	6.69^{de}
	2.0	1.00	18.33^{hi}	16.18^{b}
Setit-1	0.0	0.00	35.67^{ab}	1.37^{h}
	0.5	1.00	33.67^{abc}	2.75^{gh}
	1.0	1.00	27.67^{edf}	6.91^{ef}
	1.5	1.00	22.67^{fgh}	9.70^{de}
	2.0	1.00	20.67^{ghi}	15.25^{bc}
Non shatter	0.0	0.00	38.33^{a}	1.37^{h}
	0.5	1.00	35.67^{ab}	1.37^{h}
	1.0	1.00	33.67^{abc}	4.16^{fgh}
	1.5	1.00	32.00^{bcd}	5.54^{fg}
	2.0	1.00	29.67^{cde}	6.91^{ef}
LSD		5.38	03.38	
CV (%)		11.39	27.19	

Means in the same column with different letters are significantly different at $p \leq 0.05$

Shoot regeneration and multiplication: Shooting responses of the four varieties-in terms of days to shooting and rate of shooting-were studied under varying concentrations of BAP plus constant concentration of NAA at 1.0 mg L^{-1}. Examination of shooting responses of all varities showed that calli cultured in high concentrations of BAP required fewer days to shooting. Calli of Hirhir, Humera-1, Setit-1 and Non-Shatter cultured in MS media supplemented with 2.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA shoot on average in 16.0, 18.3, 20.7 and 29.7 days, respectively (Table 2). The shooting time of Hirhir is significantly shorter than that of the other varieties ($p \leq 0.05$). On the other hand, calli of all varieties cultured in controls and in MS media with lower concentrations of BAP resulted in delayed shooting responses. Sayem *et al*. (2010) reported similar observations in *Brassica* species.

Analyses of shooting rate (as percent of shooting) showed highest shooting rates among those cultured in MS media supplemented with higher concentrations of BAP. Calli of Hirhir, Humera-1, Setit-1 and Non-Shatter cultured in MS media supplemented with 2.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA resulted in 22.2, 16.2, 15.3 and 6.9% shooting, respectively. Shooting rate of Hirhir at 22.2% is significantly greater than that of the other varieties ($p \leq 0.05$). On the other hand, calli cultured in MS media with no supplements of BAP + NAA (controls) showed the lowest shooting rates in all of the

varieties. Examination of shooting rates of each of the varieties showed that, with the exception of Non-Shatter, calli cultured in MS media supplemented with 0.5 mg L^{-1} BAP + 1.0 mg L^{-1} NAA had significantly lower shooting rates compared to calli cultured in MS media with 2.0 mg L^{-1} BAP + 1.0 NAA ($p \leq 0.05$). Munshi *et al.* (2007) observed highest shooting response of calli cultured in shooting media supplemented with 2.0 mg L^{-1} BAP. Likewise, studies by Deepa-Singh *et al.* (2010), Du *et al.* (2000), Gangopadhyay *et al.* (1998), Sayem *et al.* (2010) and Wang *et al.* (2000) showed that higher concentrations of BAP are better in causing shooting of calli/explants.

Rooting of shoots: Rooting responses of the varities-in terms of days to rooting, root length, and rate of rooting-were studied under different concentrations of IBA plus a constant concentration of NAA at 0.5 mg L^{-1}. Data analyses shown that variations in the concentration of IBA yielded no significant difference. Hence, comparisons of rooting response were made among the four varieties. It is observed that shoots of Hirhir: took significantly fewer days to root (17.80 days); produced significantly longer mean root length (11.66 mM) and yielded significantly highest mean rooting rate (87.15%) ($p \leq 0.05$; Table 3). Non-Shatter, on the other hand, was the least responsive. Analyses of data on root number showed that Hirhir yielded significantly greater number of roots at all concentrations (Table 4, 5).

Table 3: Rooting responses of shoots of the four varieties in IBA + 0.5 mg L^{-1} NAA

	Mean rooting responses		
Varieties	Days to rooting	Length of root (mM)	Percent of rooting
Hirhir	17.80^{c}	11.66^{a}	87.15^{a}
Humera-1	21.46^{b}	8.60^{b}	66.12^{b}
Setit-1	21.66^{b}	8.20^{b}	65.86^{b}
Non-shatter	28.80^{a}	6.60^{c}	46.59^{c}
LSD	0.64	0.92	2.80
CV	4.51	14.22	5.82

Mean in the same column with different letters are significantly different at $p \leq 0.05$

Table 4: Interaction effects of different concentration of IBA and varieties on No. of roots

	Mean root No. of varities				
Sesame varieties	Concentration of IBA + NAA, mg L^{-1} 0 + 0	0.25 + 0.5	0.5 + 0.5	0.75 + 0.5	1+0.5
Hirhir	40.0^{h}	70.3^{a}	66.0^{b}	60.0^{c}	58.0^{d}
Humera-1	30.0^{l}	60.0^{c}	50.0^{f}	40.0^{h}	38.0^{i}
Setit-1	35.0^{j}	38.0^{i}	52.0^{e}	44.0^{g}	40.0^{h}
Non-shatter	20.0^{m}	44.0^{g}	40.0^{h}	36.0^{j}	32.0^{k}

Means (in the field) with different letters are significantly different at $p \leq 0.05$ (LSD = 1.66; CV = 2.21)

Table 5: Rooting response of shoots in half strength MS medium supplemented with IBA + 0.5 mg L^{-1} NAA

PGRs (mg L^{-1})		Mean rooting responses		
IBA	NAA	Days to rooting	Length of roots (mM)	Percent of rooting
0.00	0.00	20.83^{d}	3.67^{e}	52.72^{d}
0.25	0.50	18.16^{e}	14.58^{a}	76.69^{a}
0.50	0.50	22.25^{c}	11.08^{b}	74.35^{a}
0.75	0.50	24.08^{b}	8.08^{c}	67.34^{b}
1.00	0.50	26.83^{a}	6.71	62.06^{c}
LSD	0.82	1.03	3.14	
CV	4.49	11.51	5.82	

Mean in the same column with different letters are significantly different at $p \leq 0.05$

ACCLIMATIZATION

Rooted plantlets were planted on two different growing media-coco peat and soil (sand, cow dang and garden soil at a ratio of 2:1:1). The survival rates of plantlets of Hirhir, *Humera-1*, *Setit-1* and Non-shatter planted on coco peat were 66.7% (n = 12), 58.3% (n = 12), 50.0% (n = 12) and 33.3% (n = 12), respectively. Interestingly, the survival rates of plantlets of the varieties planted on soil showed similar trend-where the survival rates of Hirhir, *Humera-1*, *Setit-1* and Non-shatter were 50.0% (n = 8), 42.0% (n = 7), 33.3% (n = 6) and 25.0% (n = 4), respectively. Alam *et al.* (2009) observed similar results on the survival rate of *in vitro* produced plantlets of *Brassica*. In our study, two-third of plantlets grown on soil medium (in plastic pots) with similar proportion of sand, cow dung, and garden soil have survived.

CONCLUSION

The study showed the *in vitro* regeneration capacities of four Ethiopian sesame varieties through anther culture. It has established an efficient and reproducible protocol for *in vitro* regeneration of the varieties using anther culture. The finding is one step forward in the development and release of double haploid from which various works on the improvement the crop shall be possible. However, further works need to be carried out to investigate the ploidy levels of each variety and to establish better acclimatization condition.

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