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TISSUE CULTURE OF MICROSPERMA LENTIL (*LENS CULINARIS* MEDIK) CV. MASOOR-85

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Abstract

Various combinations of plant hormones were tested for callogenesis in different explants of microsperma lentil cultivar Masoor-85. Callogenesis was observed under dark conditions. Calli could regenerate bud primordia and a few buds developed into shoots with leaves when transferred under light conditions. Elongated shoots were cut from the base and adventitious rooting was induced to develop these into pod bearing plants in the field.

Introduction

Lentil (*Lens culinaris* Medik) is an important winter pulse crop maturing in early summer in Pakistan. The majority of lentils produced throughout the world are local land races and of the few released cultivars available to farmers almost all have originated through selection from these. Masoor-85 is one such selection in Pakistan, but the farmers rely on indigenous land races that sometimes more closely resemble wild forms than productive genotypes. The indigenous lentil is macrosperma with small dark brown seed cotyledons which are liked locally. This type is not in demand on the world market as compared to the large seeded green exotic microsperma lentil. In Pakistan lentil is grown over 63,400 hectares with an annual grain production of 28,000 tonnes @ 441 Kg per hectare (Anon., 1996). The poor yield is due to low yield potential of the cultivars, cultivation on marginal lands and low inputs of water and fertilizer.

As an adjunct to conventional breeding, plant cell and tissue culture can offer some possibilities for plant improvement. Plant regeneration from cells or callus is necessary if we are to benefit from the recent developments in gene transfer techniques. Efforts of regeneration from cells can also provide a possibility of picking somaclonal variations induced during the culturing process. Little information is available on *in vitro* cultures of *Lens culinaris* Medik. Regeneration from callus via organogenesis (Williams & McHughen, 1986), embryogenesis (Saxena & King 1987), embryo rescue from interspecific crosses (Ladizinsky *et al.*, 1985), protoplast cultures (Warkentin & McHughen 1992) have been attempted. However, these techniques are genotype specific and need efficient protocols for regeneration of microsperma lentil. The variety Masoor-85 was used for callus induction, its subculturing, shoot organogenesis, adventitious rooting of shoots and whole plant formation with a view to evolve methods for regeneration from cells and to contribute our knowledge of the processes of organogenesis in lentil.

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Materials and Methods

Seeds of lentil variety Masoor-85 were disinfected with NaOCl (10% v/v for 15 minutes) or $HgCl_2$ (0.1% w/v for 10 minutes). The latter method was more effective and was used in most of the cases. The disinfected seeds were thoroughly washed with sterile distilled water. Three to four day old germinating seedlings were found to have better regeneration potential. Explants of apical, cotyledonary nodal and stem regions were cultured in Murashige & Skoog (MS) medium (1962).

Effect of various growth regulators on growth response of shoot apices: Kinetin and BA ranging from 1-10mg/1 were used as the cytokinins both in liquid and solid media. A detailed experiment was made where shoot apices of Masoor-85 were cultured in various concentrations of K, BA, IAA, NAA, GA, alone and in combination with 2,4-D in MS liquid medium that was better than solid medium. The growth responses were recorded after five week growth period.

Effect of K and GA on formation of callus mass: The basal nodes taken as explant from germinating seedlings were routinely cultured on MS medium containing K. The callus produced was grown on various combinations of K and GA. The weight of callus formed was measured after two subcultures on the same medium.

Effect of subculturing passage and addition of lentil seed extract on shoot regenerations in callus masses: For regeneration studies, the callus cultures were transferred to 16/8h light/dark period, 4000 lux light intensity at 20 $\pm$ 2°C and allowed to grow for 22 days. Callus regeneration potential was compared for the two media. Medium-I was the same as the callussing medium except K and GA were reduced to 10 times and Medium-II in addition contained 10% lentil seed extract prepared by soaking overnight thoroughly washed seeds (10gm) in 100ml water and then heating till boiling and after that squeezing in a nylon mesh. The debris was discarded and filtrate used to make up the Medium-II. The callus masses that were grown for regeneration varied between 0.5 to 0.75 grams. The counts of differentiating callus which appeared as green dots were noted down after 22 days.

Effect of subculturing passage on shoot organogenesis: In another experiment, cotyledonary node explants, with 1/6 cotyledon attached to them were induced to form callus masses and subcultured from 1st to 10th passage. After 10th subculture the calli were transferred to six tubes (25x150mm) having 15 ml medium-II per tube. Green dots, bud primordia and elongated shoots that formed were counted after 3,6 and 8 week growth period. Two test tubes were scored for each count taken. Light and temperature conditions were the same as described above.

Effect of explant origin, callus and subsequent subculture passage on regeneration: The explants stem portions, shoot apices and cotyledonary nodes were cultured in callussing medium containing K (10mg/1) + GA (1mg/1) and the callus mass formed was subcultured after four week period in dark at 20 $\pm$ 2°C. For regenerations, subcultures were grown on Medium-II in test tubes (25x150mm) having 15ml medium and allowed to grow for 42 days in 16/8h light/dark period (light intensity 4000 lux). After this period, cultures were removed from the tubes and the number of shoot primordia (P) and emerging shoots (S) were counted. Two tubes were scored for each type of differentiation.

Table 1. Callogenesis and regeneration responses of shoot apices of Masoor-85 in various growth regulators

Treatment	Growth regulator (mg/l)	Response	Shoot regeneration potential
1.	Control	Few explants green	
2.	K (1)	Explants green with swelling	
3.	K(1)+GA (0.5)	Shoots	
4.	K(1)+IAA (0.5)	Green buds	+
5.	K(2.5)	Green buds	++
6.	K(2.5)+GA (0.5)	Shoots	
7.	K(2.5)+IAA (0.5)	Green buds	++
8.	K(5)	Green buds	++
9.	K(5)+GA (0.5)	Green buds+shoots	++
10.	K(5)+IAA (0.5)	Green buds	++
11.	K(7.5)	Green buds	++
12.	K(7.5)+IAA (0.5)	Green buds+shoots	++
13.	K(10)	Green buds	++
14.	K(10)+GA (0.5)	Green buds+shoots	+++
15.	K(10)+IAA (0.5)	Green buds	+
16.	K(2.5)+NAA (0.5)	Green buds	++
17.	BA(2.5)+GA (0.5)	Green buds+shoots	+
18.	BA(2.5)+IAA (0.5)	Green buds	
19.	K(1)+2,4-D (2.5)	Swelling of explants	
20.	BA(1)+2,4-D (2.5)	Swelling of explants	
21.	2,4-D (2.5)	Swelling of explants + callus at cut ends	

+ minimum, ++ medium, +++ maximum : shoot regeneration potential.

Table 2 Effect of K (mg/1) and GA (mg/1) combination on lentil callus growth* (gm).

	GA		
K	0.1	0.5	1.0
1	0.17	0.22	0.19
5	0.33	0.28	0.44
10	0.34	0.51	0.84

*Explant were basal nodes from germinating seedling.

Data taken two month after inoculation of second subculture callus.

Effect of exposure time to auxins on adventitious rooting: The possible effect of auxin exposure time was studied for adventitious root induction in excised or cut shoots. The shoots (5-7 cm long) from regenerated cultures were kept in auxin mixture (IBA 0.008% + NAA 0.002%) for a period of 1 to 6 days. Fifty shoots were used for each treatment. After exposure to auxin for required time period, they were transferred to auxin free system and grown in plastic pots of 15 cm long + 18 cm diameter containing soil, sand, peat mixture (1:1:1 w/w). The survived shoots were observed for the number of roots developed after one month growth for each exposure time period. During the root establishment phase, the shoots were kept under the polythene cover to keep humidity and water sprays were also made 7-8 times in a day.

Results

Callus was induced in lentil explants taken from either apical, cotyledonary or stem portions. Explants taken from one week or older seedlings did not show good response towards callusing as the germinating seedlings. Callus was multiplied by subculturing and maintained for more than one year. The callus upon subculturing, after two or three passages, became more friable and increased rapidly in volume.

Effect of various growth regulators on growth responses of shoot apices: The result of the effect of various growth regulators on callogenesis and organogenesis from shoot apex explant are depicted in Table 1.

In the initial experiment K was used at 2.5 mg/l in the liquid medium only. In this medium apical shoot explants grew, proliferated and developed green buds. Green buds and shoots also developed from the shoot apices with GA treatment. However, with K (10mg/l) + GA (0.5mg/l) combination, the shoot buds formation and shoot development proved to be the best. It was also observed that the growth responses in liquid medium were better as compared to that in the agar solidified medium. Shoot buds and shoots were also developed in K + IAA, BA + GA combinations, while 2,4-D had a tendency to form callus.

When explants were cultured on various media containing 2,4-D they produced callus in the presence of BA or K. The best combination of growth regulators for callus formation was BA at 4mg/l with 2,4-D at 1mg/l for subculturing the callus masses. Light green callus originated from the explant as well as from cell suspension cultures derived callus when plated onto agar medium. The callus was comparatively compact at the begining but on subsequent subculturing it became pale white and friable. The callus formed from the embryonic axis tissue was pale white and slow growing and it was loosely spread over the medium. After three to four subculture passages, all calluses derived from different explants looked similar in form, irrespective of the origin of the explant (data not shown).

Effect of K and GA on regeneration from callus mass: In dark the kinetin and GA combinations induced formation of callus (Fig. 1A). Maximum callus mass was produced at K (10 mg/l) and GA (1 mg/l) combination (Table 2). The callus produced bud primordia when shifted under light conditions. Differentiation of shoot buds was initiated as green dots (Fig. 1B) which eventually produced shoot buds (Fig. 1C).

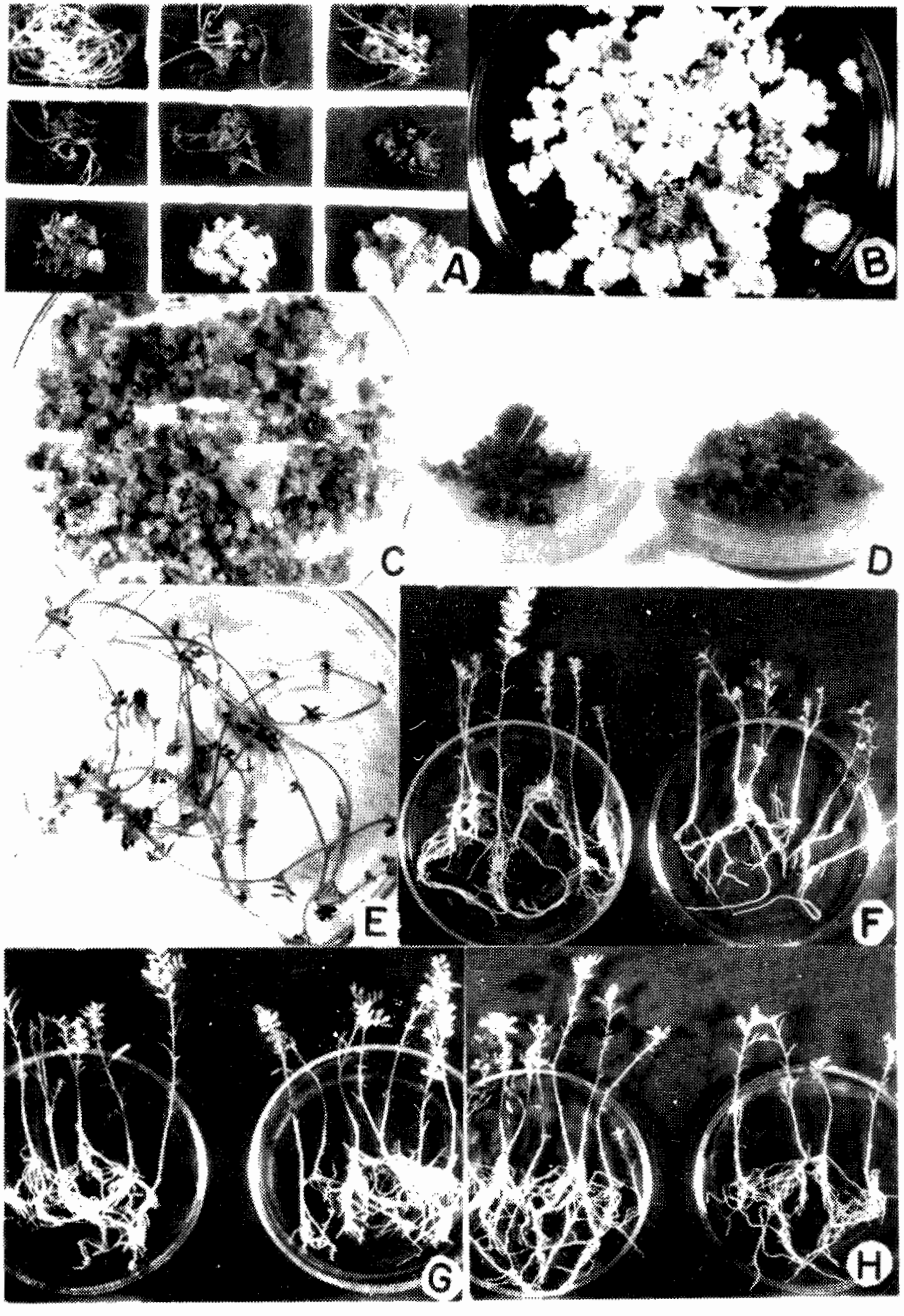

Fig. 1A. Lentil callogenesis in dark. B, callus pieces in light starts regeneration as green dots. C, development of shoot buds. D, Shoot primordia. E, elongation of shoots. F, adventitious rooting in excised shoots exposed to auxin for one day (left), two day (right); G, 3 day (left), 4 day (right); H, 5 day (left), 6 day (right).

Table 3. Effect of subculturing passage and medium on shoot primordium formation in callus masses after 22 day growth.

Sub-culture passage No.	Counts of differentiating shoot of primordia Medium-I	Medium-II
1.	4	7
2.	102	153
3.	129	173
4.	126	172
5.	65	187
6.	81	127
7.	163	253
8.	164	136
9.	116	200
10.	100	214

Explant: cotyledonary nodes with 1/6th cotyledon.
Medium-I: MS+K (10mg/1)+GA(1mg/1), Medium-II: Mddium-I + 10% seed extract.
Subculture: Passage period was 30 days.

Effect of subculturing and media on shoot regeneration: Shoot primordium formation (Fig.1D) and elongation of shoots (Fig.1E) was studied in Medium I and Medium II (Table 3). The regeneration of shoots from the callus derived from cotyledonary nodes with 1/6th cotyledons attached started in the first subculture and was maximum in the 7th subculture stage where number of green dots were 253 in the seed extract containing medium-II. The minimum number of primordia counted were 4 in the first culture in medium I without seed extract. The callus from first culture had the least differentiated regions. Maximum shoot primordia developed in the most proliferative friable callus mass irrespective of the subculturing passage. However, sudden decrease in number of differentiating shoot primordia counts in 5th and 6th subcultures was because of more non-regenerating callus and overall callus proliferation was less for unknown reasons as compared to 7th and 8th subcultures. The differentiating points first transformed into shoot primordia and later into elongated shoots (Fig. 1E). The callus surface regenerated to maximum capacity in about one month time. However, shoot buds remained proliferating with the elongation of shoots for 3-4 subcultures in the shoot regeneration medium.

Effect of subculturing passage on shoot organogenesis: In another experiment the calli with differentiating green dots were subcultured in Medium II. After six week, data on number of differentiating green dots, shoot primordia and the shoots developed per callus mass were taken (Table 4). Under these growth conditions the maximum number of green dots (305) and shoot primordia (396) were obtained for the 8th subculture callus. The texture of callus was important as the most proliferative callus mass gave

Table 4. Effect of callus subculturing passage on shoot organogenesis.

Subculture passage No.	Differentiating green dots (No)	Shoot primordia (No)	Shoots developed
1	179	117	4
2	-	49	6
3	16	320	2
4	140	254	-
5	109	296	-
6	108	212	1
7	187	372	-
8	305	396	-
9	299	192	8
10	255	316	-

Explant: cotyledonary nodes with 1/6th cotyledon, Medium: MS+K (10mg/1)+GA (1mg/1) + 10% seed extract, Green dots were counted after 3 week, shoot primordia after 6 week and shoots after 8 week period.

maximum regenerations. The green dots ultimately formed shoot primordia which usually developed single shoot. Multiple shoots were rarely formed.

Effect of callus subculture passage and explant origin on shoot regeneration: The shoot regeneration response was not much affected by the source of explant callus, neither by the age of callus nor by the subculturing passage. The fast growing callus gave maximum regenerations for shoot primordia. The maximum number of shoot bud primordia were 103 in the shoot apical derived calli in the 5th subculture. The minimum number of shoot bud primordia were 31 in 8th subculture of cotyledonary node explant callus, some of which elongated into shoots. The maximum number of grown shoots were 26 in stem callus in the 4th and 10th subcultures. The minimum number of shoots were 6 in stem callus in third subculture (Table 5).

Rooting in shoot cuttings: The overal effect of auxin exposure on adventitious rooting has been depicted in Table 6. It is clear from the data that 4 day exposure of auxins to the shoot bases seems to be optimum for rooting on the basis of shoot survival (30.0%), average number 16.25% and mean length 6.47%. (Fig.1 F-H).

Discussion

Leguminosae species in general and grain legumes in particular are difficult to regnerate *in vitro* (Malik & Saxena 1991). For efficient plant regeneration it is necessary to define specific requirements of a species and even genotypes (Dhir *et al.*, 1992), because it is not workable to give generalized culture medium for achieving totipotency in all plants or even in all cells of the same kind. Further the kind and balance of growth regulators needed for various morphogenetic responses vary from tissue to tissue (Fakhrai & Evans, 1989) and cell to cell (Barcelo *et al.*, 1991) depending on their metabolic status. Not much work has been done on tissue culture of

Table 5. Effect of callus subculture passage and explant origin on regeneration after 42 day growth.

Subculture passage No.	Mode of regeneration	Explant source Stem	Shoot apex	Cotyledonary node
3	P	61	70	66
	S	6	20	19
4	P	96	59	81
	S	26	14	17
5	P	90	103	87
	S	15	15	22
8	P	56	75	31
	S	18	15	5
9	P	59	65	69
	S	8	10	7
10	P	96	59	81
	S	26	14	17

P = Shoot primordia, S = Elongated shoot.

lentil especially microsperma. This work describes the shoot regenerations from explants and calli of a specific genotype Masoor-85 of microsperma lentil. Adventitious regenerations directly from explants of the seedling of less than one week gave a limited number of propagules. This may be increased manyfold by an intervening callus phase, however, at the risk of undesirable variants. Kinetin or BA alone in light conditions is sufficient to induce few shoot buds without an intermediate callus stage. Higher K (10 mg/1) with GA (1 mg/1) was required for induction of callus and shoot organogenesis, while low quantities of K (1 mg/1 and GA (0.1 mg/1) were sufficient for subsequent continuous multiplication of buds. No adventitious shoot induction

Table 6. Effect of auxin* exposure time on adventitious root formation.

Exposure time (day)	Shoot survival (%)	Average No of roots	Mean length of roots (cm)
1	12	24.60	5.92
2	16	6.60	3.90
3	18	17.00	5.60
4	30	16.25	6.47
5	22	16.25	6.30
6	8	18.25	6.10

*Auxin was a mixture of IBA + NAA (0.008% + 0.002%)

occurred in the absence of cytokinins. Shoots were also regenerated directly from the meristems of seedlings on the shoot regeneration medium. Meristems remained alive and growing even in basal medium (unreported work). Cytokinins and auxins produce callus. Most proliferation of callus cells was when 2,4-D was used as an auxin. But the best regenerating callus was obtained with K and GA in dark. In the initial cultures the callus was slow growing but with subsequent subculturing, it increased in volume. Callus cultures could be maintained for extended periods by subculturing at 2-4 week intervals under the same culture conditions and therefore, represent a conveninet form for the longer maintenance potential.

For organogenesis, callus definitely needs light. After two weeks in light, green spots appeared on callus followed by shoot bud formation. Adventitious buds were small and embedded in callus mass. Typically, GA had a stimulatory effect on the proliferation and also on growth of adventitious buds. Although organogenesis was relatively high in callus cultures, only a few buds elongated into shoots. Similar results have been found by other workers (Bates *et al.*, 1992; Bejoy & Hariharan 1992). In experiments with supplementation of various auxin, gibberellin and cytokinin to the medium supported bud formation and their elongation in callus at the time of callus transfer to light conditions. The shoot primordia formation was extensive but their potential to form and elongate as shoots was limited. Some shoots became swollen and vitrified. The number of buds in individual calli and their elongation also varied in different experiments. In order to produce shoots of quality and size suitable for rooting *in vitro* it is desirable to have elongated shoots that are rhizogenically competent and capable of quickly resuming photoautotrophic growth *ex-vitro*. Lentil species have in general little root organogenic potential for newly formed shoots (Williams & McHughen 1986) and so it was in experiments with Masoor-85. In some other species, plant regeneration via bud induction is limited by poor rooting (Budimir & Vujicic, 1992). Kind of auxin, auxin exposure time to bases of shoot cuttings for root induction, proper connections between root and shoot system, humidity, environmental temperature, all contribute to the survival of the regenerated shoots in lentil. Humidity is essential during the root establishment period, otherwise shoots wilt and dry. Acclimatization to the soil environment is time dependent and requires the production of new leaves initiated in the new environment. Further investigation is needed to enhance the production of elongated shoots and for the shoots that are not able to survive as they fail to initiate the formation of root system.

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