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REGENERATION OF LIQUORICE (*GLYCYRRHIZA GLABRA* L.) FROM CULTURED NODAL SEGMENTS

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SUMMARY

A protocol has been established to propagate local cultivar of *Glycyrrhiza glabra* (L.) through indirect and direct *in vitro* organogenesis from nodal segment explants excised from 4-5 months-old plants. In the present study, induction medium $B_5$2D (B_5 + 2.0 mgl^{-1} 2, 4 D + 20.0 gl^{-1} sucrose + 7.5 gl^{-1} agar) induced callusing in higher frequencies (65.93 %). Culture medium B_5.5B.5N (B_5 + 0.5 mgl^{-1} BA + 0.5 mgl^{-1} NAA + 20.0 gl^{-1} sucrose + 7.5 gl^{-1} agar) was found to be more responsive for shoot proliferation (94.12 %), shoots per explant (9.32) and mean shoot length (5.20 cm). Among rooting media B_5.5I (B_5 + 0.5 mgl^{-1} IBA+ 10.0 gl^{-1} sucrose + 7.5 gl^{-1} agar) proved to be superior for higher root proliferation (84.92%) and mean root length (2.13 cm). Regenerated plantlets were established successfully in the field after hardening.

***Keywords: Glycyrrhiza glabra*, nodal segment culture, organogenesis, plantlet regeneration.**

INTRODUCTION

Liquorice (*Glycyrrhiza glabra* L.) is one of the important plants in Indian system of medicine. In India it is known as "Mulhati". It is widely distributed in the world from 5 ^{0} W to 100 ^{0} E longitudes and 20^0 to 50 ^{0} N latitude. It is also recorded to be distributed in warm parts of Mediterranean region of North Africa, Spain, Italy, Greece and Syria. This plant can be cultivated in plains of India particularly in Haryana and Gujarat but is actually cultivated in very small scale. India requires 5000 to 10,000 tonnes of dry roots annually which is imported from Afghanistan and Iran. The principle constituent of liquorice is sweet tasting triterpenoidal saponin, glycyrrhetic acid and D-glucuronic acid (Elagamal *et al.* 1965). Besides, yellow colouring matter in roots has been identified as iso-liquiritin (Puri and Seshadri 1954) a flavonoid. Lycorine has been isolated as only alkaloid from liquorice roots (Humber *et al*. 1954). Glycyrrhetic acid has been shown to possess anti-inflammatory activity and is used in treatment of gastric ulcer, but it causes oedema and hypertension in some cases (Finney and Tarnocky 1960). Hence D-glycyrrhized liquorice extract is now used as an important substance for treatment of gastric and duodenal ulcer (Gujral *et al*. 1959).

Although, traditionally *Glycyrrhiza glabra* is propagated through seed and stolon cuttings, commercial cultivation is restricted due to limited seed set and poor seed viability. Furthermore, the conventional method of propagation is rather very slow and only 40-50 % of the cuttings get established in the field. To cater the growing demand for pharmaceutical based industries, it is essential to multiply this species through tissue culture. Efforts have been made to produce regenerable cultures of liquorice *via in vitro* morphogenesis using explants such as nodal segments (Dimitrova *et al*. 1994, Kukreja 1998,

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Patel and Shah 2007), shoot tips (Shah and Dalal 1980), seedlings (Shah and Dalal 1980), auxiliary bud (Thengene *et al.* 1998), cell suspension (Yoo and Kim 1976, Hayashi *et al.* 1988, Arias-Castro *et al.* 1993), shoot apex (Yoo and Kim 1976), stem cuttings (Hayashi *et al.* 1988), hypocotyl (Hayashi *et al.* 1988) and root (Hayashi *et al.* 1988). However, the regeneration frequencies in all above experiments were found to be low to moderate. In the present study, an attempt has been made to develop an effective protocol for shoot proliferation from the nodal segments of *Glycyrrhiza glabra* cultivars to generate highly responsive protocol for use *in vitro* propagation.

MATERIALS AND METHODS

Plant material: Local cultivar of liquorice was chosen for the culturing of the nodal segment. Explants were collected from 4-5 months old plants planted at Bahadri Farm, KNK College of Horticulture, Mandsaur, India.

Culture media: To begin with, a preliminary experiment was conducted to find out better *in vitro* response among the two basal media *viz*: MS (Murashige and Skoog 1962) and B_5 (Gamborg 1968). In this investigation, B_5 basal medium was found to be more responsive as compared to MS medium. Subsequently, basal B_5 medium was used for all the experimentations. All initial culture media were made using readymade basal B_5 salts and vitamins (HiMidia®) and three sets of plant growth regulators. In first set: three different auxins, *namely*: 2, 4-D, NAA and 2, 4, 5-T (alone), in second set: three diverse cytokinins *viz*: BAP, Kinetin and TDZ (alone) and in third set: different cytokinins (BA, Kn and TDZ) in combination with an auxin (NAA) in varying concentrations were supplemented to screen the best *in vitro* response. All initial media were supplemented with 20.0 gl^{-1} sucrose 7.5 gl^{-1} agar powder and pH was adjusted to 5.8 ± 0.1.

Surface sterilization, excision and plating technique of nodal segment: The indeterminate shoots with nodes were collected from the middle portion of field grown plants. The nodal segments were pretreated with 2 % Tween 20 (v/v) for 20 min. and 70% (v/v) ethanol for 1 min. To find out an effective surface sterilizing method, a treatment of aqueous solution of two different surface sterilizing agents *i.e.* $HgCl_2$ and $Ca(OCl)_2$ alone in different concentrations as well as in combination with Bavistin® (BASF, Germany) in varying concentrations for different durations with initial vacuum of 100 psi were tested. Finally, the segments were subjected to 4-5 rinsing with sterile double distilled water.

Culture conditions: Baby food bottles/culture tubes containing cultures sealed with Lab film (Parafilm®) were incubated under complete darkness at 25±2 °C for one week. Later *in vitro* cultured nodal segments were subjected to photoperiod regime of 16 h light and 8 h dark cycle at an intensity of 1200-lux luminance provided by PAR lamps.

Regeneration of plantlets: After 4-5 weeks of initial culturing, nodal segments followed either direct plant regeneration (auxiliary bud proliferation) or indirect morphogenesis (*via* callus formation). Shootlets obtained from direct proliferation were transferred to elongation medium B_5 with 1.0 mgl^{-1} GA_3, 15.0 gl^{-1} sucrose and 7.5 gl^{-1} agar. However, calli were subcultured again on the initial medium after four weeks of culture. Cultures were subjected to 25±2 °C temperature and photoperiod regimes of 60m mol m^{-2} s^{-1} luminance provided by cool fluorescent tubes for 16 hr.

In vitro rooting of regenerants: At times root formation was not obtained on regeneration medium, plantlets were subsequently transferred to B_5 rooting medium supplemented with varying strength of IBA, NAA, BA and Kn (alone as well as in combinations), 10 gl^{-1} sucrose and 7.5 gl^{-1} agar.

Hardening of regenerated plants: Plants uprooted from cultures and thoroughly washed with running tap water to remove the adhering agar were planted in 2.5 cm root trainers filled with 1:1:1 sand, soil and FYM sterilized mixture. Root trainers with transplanted plants were placed under 30±2°C and 70 ± 5% RH for 15-20 days for acclimatization. Acclimatized plants then were transferred to net house for 30 days for hardening before transplanting to the field.

Experimental design and analysis of data: Completely Randomized Design (CRD) was adopted to find out the significance of different culture media combinations.

Each treatment was consisted of two replications. Per replication approximately 80-100 nodal segments were cultured. The data were analyzed as per method suggested by Snedecor and Cochran (1967).

RESULTS

There were highly significant ($p<0.01$) differences among the response of different surface sterilizing and antifungal agents. The highest percentage of survived nodal segment aseptic culture was recorded with 1% Bavistin in combination with 0.1% $HgCl_2$ for 10 min. (data not presented). Consequently, this surface sterilization combination was used throughout the experiment.

During present investigation, three different sets of plant growth regulators were added to fortify B_5 basal media for culturing nodal segment of liquorice. Depending upon the nature of different culture media combinations, cultured nodal segments followed either direct or indirect pathway of plant regeneration. In direct approach, plantlets were regenerated on explant surface directly without callus formation (*via* auxiliary bud proliferation); and in indirect mode plantlets originated *via* callus formation (indirect organogenesis). In most of the cases, shootlets were developed directly from the meristematic zones of cultured nodal segments (Fig.1 A-G). Shoot formation from cultured nodal segments started approximately 7-30 days from initial culturing and proliferated 1-9 shoots (Fig.1A-G).

Plantlet also regenerated *via* organogenesis from the callus surface (Fig.1H-L). The first response of cultured explants was similar after 4 -7 days and mostly independent from explant and culture media. All explants became swollen and no callus proliferation was evident during first few days. Callus proliferation usually started from the portion in contact with the medium and spread upward after 2 weeks of culture (Fig.1H). Initiated callus tissue developed distinct phenotypes. These pheno-variants were rough, hard, dense and glossy reflecting different developmental potentials. Plantlet regeneration occurred routinely (Fig.1 I-L) after sub culturing of organogenic calli. Initiation of root started after 10-15 days of transferring of shootlets in rooting medium (Fig.1 M). Regenerated plantlets (Fig.1 O) were subjected to phenotypic evaluation. Although the traits were not scored quantitatively, regenerated plants appeared phenotypically normal and true to the type.

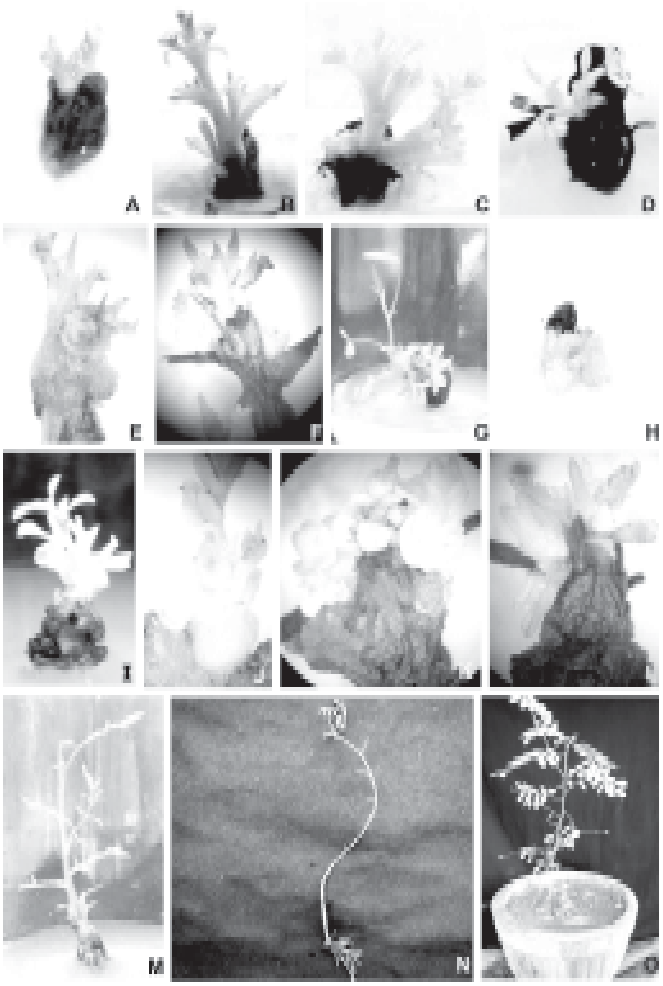

Figure 1. Plant regeneration from nodal segments: A. Initiation of shoot after 7-10 days in culture; B. Formation of single shoot after 20-25 days in culture; C. Formation of two shoots after 20-25 days in culture; D. Initiation of multiple shoots after 7-10 days in culture; E. Proliferation of multiple shoots after 10-15 days in culture; F. Proliferation of multiple shoots after 20-25 days in culture G. Formation of multiple shoots after 30-40 days in culture H. Formation of callus after 15-20 days in culture; I. Regenerated plantlets after 30-40 days in culture; J. Initiation of multiple shoots after 10-12 days in culture; K. Formation of multiple shoots after 15-20 days in culture; L. Formation of multiple shoots after 25-30 days in culture; M. Elongated plantlets after 40-50 days in culture; N. *In vitro* rooting of regenerants; and O. Plants in pots after 60-70 days of initial culturing.

Nodal segments of Local cultivar of liquorice were cultured on different fortifications of B_5 medium. The analysis of variance (Table 1-4) revealed highly significant ($p<0.01$) differences among the response of different culture medium combinations for overall callus induction, shoot proliferating efficiency, number of shoots per explant, mean shoot length, root proliferating efficiency, number of roots and mean root length. It indicates the presence of considerable amount of variability amongst the different culture media combinations. Furthermore, the response of culture media combinations varied significantly ($p <0.01$) for the various *in vitro* attributes. However, non-significant differences were also observed among different culture media combinations fortified with auxins alone for number of shoots per explant and mean shoot length.

Effects of different auxins in varying concentrations on *in vitro* are presented in Table 1. Higher callus initiation with culture media fortified with 2,4-D $B_5$2D (65.93 %) and B53D (62.92 %) and low callus initiation on media devoid of 2,4-D such as B_5 1N (9.17%) reveal that 2.0-3.0 mgl^{-1} 2,4-D induces callus in higher frequencies (62 -65%). The size of callus also enlarged with the increased level of 2,4-D however, levels above 3.0 mgl^{-1} reduced the response. At the concentration of 5.0 mgl^{-1} most of the calli turned black with apparent cell mortality. Culture medium altered with NAA and 2, 4, 5-T produced calli in lower to moderate frequencies depending upon the concentrations. On the other hand, NAA in the range of 3.0-4.0 mgl^{-1} proved to be remarkably superior for shoot proliferating efficiency since culture media $B_5$3N (21.77 %) and $B_5$4N (20.97

Table 1. Effects of different auxins (alone) in varying concentrations on *in vitro* response of nodal explant cultures in liquorice.

Culture Media	Auxins	Conc. (mgl^{-1})	Callus induction (%)	Shoot proliferating explant (%)	No. of shoots per responding explant	Mean shoot length (in cm)
B_5.1D	2,4-D	0.1	12.38^{fg} (20.54)	3.56^{g} (10.66)	1.04 (5.85)	0.58 (4.34)
B_5.5D		0.5	14.59^{f} (22.44)	4.01^{g} (11.39)	1.08 (5.96)	0.96 (5.55)
B_5D		1.0	28.36^{e} (32.16)	9.28^{e} (17.69))	1.09 (5.99)	1.09 (5.99)
$B_5$2D		2.0	65.93^{a} (54.27)	14.09^{c} (22.01)	1.10 (6.01)	0.79 (5.07)
$B_5$3D		3.0	62.92^{a} (52.48)	16.03^{c} (23.57))	1.12 (6.07)	0.70 (4.77)
$B_5$4D		4.0	48.70^{b} (44.24)	13.93^{c} (21.89))	1.06 (5.91)	0.61 (4.39)
$B_5$5D		5.0	29.84^{e} (33.09)	12.97^{cd} (21.06)	1.03 (5.82)	0.59 (4.36)
B_5.1N	NAA	0.1	9.17^{g} (17.54)	4.12^{g} (11.59)	1.06 (5.91)	0.73 (4.88)
B_5.5N		0.5	12.09^{g} (20.26)	4.86^{g} (12.67)	1.07 (5.93)	1.18 (6.23)
B_5N		1.0	27.86^{e} (31.82)	9.45^{de} (17.87	1.08 (5.96)	1.21 (6.31)
$B_5$2N		2.0	47.38^{b} (43.47)	15.30^{c} (23.01)	1.11 (6.04)	0.91 (5.47)
$B_5$3N		3.0	45.92^{bc} (42.64)	21.77^{a} (27.78	1.20 (6.28)	0.88 (5.36)
$B_5$4N		4.0	41.79^{cd} (40.25)	20.97^{a} (27.21	1.14 (6.13)	0.84 (5.23)
$B_5$5N		5.0	38.92^{d} (38.57)	16.79^{b} (24.17)	1.02 (5.79)	0.63 (4.55)
B_5.1T	2,4,5-T	0.1	11.05^{g} (19.38)	2.68^{g} (9.32)	1.04 (5.85)	0.37 (3.47)
B_5.5T		0.5	10.82^{g} (19.11)	3.60^{g} (10.88)	1.09 (5.98)	0.55 (4.24)
B_5T		1.0	12.08^{g} (20.30)	4.02^{g} (11.53)	1.06 (5.91)	0.64 (4.57)
$B_5$2T		2.0	14.93^{f} (22.67)	5.33^{fg} (13.31)	1.08 (5.96)	0.66 (4.65)
$B_5$3T		3.0	26.78^{e} (31.15)	15.19^{c} (22.93))	1.05 (5.87)	0.89 (5.39)
$B_5$4T		4.0	45.57^{c} (42.44)	12.87^{d} (20.99)	1.06 (5.91)	0.81 (5.16)
$B_5$5T		5.0	38.62^{d} (38.40)	8.92^{ef} (17.35)	1.03 (5.82)	0.72 (4.86)
Mean			**30.74 (33.65)**	**10.46 (18.86)**	**1.07 (5.93)**	**0.77 (5.03)**
CD(0.05)			**4.24**	**3.69**	**NS**	**NS**

1 Figures in parenthesis are transformed values (Arc-sine transformation).

1 Values within column followed by different letters are significantly different at 5% probability level.

1 NS: Non-significant

Table 2. Effects of different cytokinins (alone) in varying concentrations on *in vitro* response of nodal explant cultures in liquorice.

Culture Media	Cytokinins	Conc. (mgl^{-1})	Callus induction (%)	Shoot proliferating per explant (%)	No. of shoots per responding explant	Mean shoot length (in cm)
B_5.1B	BA	0.1	5.12^{c} (13.07)	84.92^{ab} (67.14)	2.98^{b} (9.84)	2.18^{b} (8.46)
B_5.5B		0.5	6.29^{c} (14.48)	86.28^{a} (68.26)	4.77^{a} (12.51)	3.13^{a} (10.17)
B_5 B		1.0	8.46^{b} (16.86)	84.29^{b} (66.66)	3.68^{a} (11.00)	1.92^{b} (7.95)
$B_5$2 B		2.0	11.27^{a} (19.57)	70.89^{g} (57.34)	2.26^{b} (8.61)	2.59^{b} (9.24)
$B_5$3 B		3.0	12.32^{a} (20.52)	62.30^{i} (52.11)	1.91^{b} (7.93)	1.92^{b} (7.95)
$B_5$4 B		4.0	9.02^{b} (17.42)	56.32^{j} (48.61)	1.78^{b} (7.65)	1.81^{b} (7.72)
$B_5$5 B		5.0	8.79^{b} (17.15)	55.29^{j} (48.01)	1.08^{b} (5.96)	1.76^{b} (7.61)
B_5.1kn	Kinetin	0.1	6.82^{c} (15.07)	82.73^{bc} (65.43)	2.43^{b} (8.92)	2.89^{ab} (9.73)
B_5.5kn		0.5	7.36^{c} (15.72)	84.09^{b} (66.47)	3.83^{a} (11.24)	2.48^{b} (9.05)
B_5kn		1.0	8.46^{b} (16.87)	78.33^{de} (62.23)	3.34^{ab} (10.49)	2.90^{b} (8.69)
$B_5$2kn		2.0	9.43^{b} (17.85)	70.23^{g} (56.91)	2.66^{b} (9.34)	1.75^{b} (7.56)
$B_5$3kn		3.0	11.39^{a} (19.67)	56.38^{ij} (48.64)	2.33^{b} (8.76)	1.45^{b} (6.87)
$B_5$4kn		4.0	6.24^{c} (14.42)	54.08^{jk} (47.32)	1.25^{b} (6.40)	1.28^{b} (6.47)
$B_5$5kn		5.0	4.89^{c} (12.69)	52.39^{bc} (46.35)	1.16^{b} (6.17)	0.98^{b} (5.66)
B_5.1Td	TDZ	0.1	4.86^{c} (12.71)	84.72^{b} (66.97)	3.51^{a} (10.70)	3.12^{a} (10.16)
B_5.2Td		0.2	5.48^{c} (13.53)	81.92^{c} (64.82)	3.40^{a} (10.61)	2.82^{b} (9.62)
B_5.3Td		0.3	6.28^{c} (14.50)	78.04^{e} (62.03)	3.00^{b} (9.96)	2.45^{b} (8.89)
B_5 .4Td		0.4	8.39^{b} (16.82)	80.71^{cd} (63.9)	2.46^{b} (9.01)	2.38^{b} (8.83)
B_5.5Td		0.5	10.29^{ab} (18.69)	77.45^{e} (61.62)	2.18^{b} (8.47)	1.89^{b} (7.84)
B_5Td		1.0	9.32^{b} (17.75)	74.12^{f} (59.39)	2.16^{b} (8.44)	1.76^{b} (7.57)
B_5 2Td		2.0	7.79^{bc} (16.13	66.89^{h} (54.85)	1.58^{b} (7.20)	2.22^{b} (7.59)
Mean			**8.01 (16.43)**	**72.49 (58.08)**	**2.55 (9.18)**	**2.09 (8.30)**
CD(0.05)			**3.18**	**2.47**	**2.30**	**2.06**

1 Figures in parenthesis are transformed values (Arc-sine transformation).

1 Values within column followed by different letters are significantly different at 5% probability level.

%) performed higher for this trait. However, beyond this concentration, ratio of non-morphogenic calli increased subsequently. Auxins 2, 4-D (16.03 %) and 2, 4, 5-T (15.19 %) responded poorly for this attribute.

In vitro response of different cytokinins is presented in the Table 2. Varying concentrations of BAP, Kinetin and TDZ supplemented in the medium exhibited very poor callus induction. For shoot proliferating ability, culture media $B_5$5B (86.28 %) closely followed by $B_5$1B (84.92 %) proved remarkably superior as compared to other media combinations. The shootlets of higher length were recovered from culture media $B_5$5B (3.13cm), $B_5$1Td (3.12 cm) and $B_5$1kn (2.89 cm).

Low to moderate results from initial studies to establish plant growth regulator type and concentrations revealed that auxins as well as cytokinins alone are not effective for achieving higher *in vitro* response. Therefore, combinations of an auxin and a cytokinin were considered for achieving the best *in vitro* response.

Combined effects of different added auxins and cytokinins in varying concentrations on various culture phases are presented in Table 3. Maximum callus induction was documented by induction media $B_5$3N5B (38.21 %) followed by $B_5$4N.5B (35.79 %). Inoculation media $B_5$5B5N (94.12 %), B_5B5N (93.75 %), $B_5$1Td.5N (92.37 %) and $B_5$5Kn.5N (91.66 %) proved remarkably

Table 3. Combined effects of different auxins and cytokinins on *in vitro* response for cultured nodal segments in liquorice.

Culture Media	Plant Growth regulators mgl^{-1}				*In vitro* response			
	NAA	BAP	TDZ	Kinetin	Callus induction (%)	Shoot proliferating explant (%)	No. of shoots per responding explant	Mean shoot length (in cm)
B_5.5B.N	0.5	0.5	-	-	19.02^{g} (25.84)	94.12^{a} (75.96)	9.32^{a} (17.69)	5.20^{a} (13.15)
B_5B.5N	0.5	1.0	-	-	22.59^{e} (28.35)	93.75^{a} (75.71)	6.98^{b} (15.20)	3.28^{b} (10.36)
$B_5$2B.5N	0.5	2.0	-	-	16.39^{g} (23.83)	89.46^{b} (71.10)	3.79^{bc} (10.96)	2.98^{b} (9.84)
$B_5$3B.5N	0.5	3.0	-	-	14.57^{h} (22.42)	80.19^{e} (63.55)	2.85^{c} (9.62)	2.48^{b} (8.97)
$B_5$4B.5N	0.5	4.0	-	-	12.92^{hi} (21.01)	69.23^{h} (56.29)	2.03^{c} (8.16)	1.97^{b} (7.91)
$B_5$5B.5N	0.5	5.0	-	-	10.27^{i} (18.67)	62.54^{h} (52.24)	1.78^{c} (7.50)	1.49^{b} (6.95)
B_5.5Kn.5N	0.5	-	-	0.5	19.09^{fg} (25.87)	91.66^{ab} (73.30)	7.25^{ab} (15.53)	4.79^{a} (12.54)
B_5Kn.5N	0.5	-	-	1.0	20.19^{f} (26.68)	90.06^{b} (71.62)	6.95^{b} (15.25)	3.20^{b} (10.24)
$B_5$2Kn.5N	0.5	-	-	2.0	22.35^{ef} (28.19)	89.66^{b} (71.24)	3.56^{c} (10.78)	2.78^{b} (9.53)
$B_5$3Kn.5N	0.5	-	-	3.0	11.92^{i} (20.14)	85.23^{cd} (67.39)	3.08^{c} (10.08)	1.45^{b} (6.85)
$B_5$4Kn.5N	0.5	-	-	4.0	12.87^{i} (20.98)	82.38^{e} (65.18)	2.13^{c} (8.38)	1.28^{b} (6.46)
$B_5$5Kn.5N	0.5	-	-	5.0	11.83^{i} (20.04)	80.20^{e} (63.55)	2.03^{c} (8.18)	1.08^{b} (5.92)
B_5.1Td.5N	0.5	-	0.1	-	11.02^{i} (19.32)	92.37^{a} (74.14)	6.97^{b} (15.25)	3.84^{ab} (11.19)
B_5.2Td.5N	0.5	-	0.2	-	15.79^{gh} (23.39)	87.72^{bc} (69.48)	5.28^{b} (13.26)	2.65^{b} (9.28)
B_5.5Td.5N	0.5	-	0.5	-	13.64^{h} (21.65)	83.46^{de} (65.98)	3.79^{b} (11.14)	2.39^{b} (8.87)
B_5Td.5N	0.5	-	1.0	-	17.98^{g} (25.06)	79.98^{e} (63.43)	3.25^{c} (10.30)	1.78^{b} (7.58)
B_5 2Td .5N	0.5	-	2.0	-	21.08^{f} (27.31)	75.84^{f} (60.55)	2.92^{c} (9.69)	1.23^{b} (6.32)
B_5N.5B	1.0	0.5	-	-	29.18^{d} (32.67)	73.33^{f} (58.89)	1.83^{c} (7.67)	2.30^{b} (8.67)
$B_5$2N.5B	2.0	0.5	-	-	32.98^{bc} (35.01)	60.89^{h} (51.27)	1.59^{c} (7.14)	1.65^{b} (7.31)
$B_5$3N.5B	3.0	0.5	-	-	38.21^{a} (38.16)	40.20^{i} (39.33)	1.27^{c} (6.43)	1.45^{b} (6.87)
$B_5$4N.5B	4.0	0.5	-	-	35.79^{ab} (36.72)	38.79^{ij} (38.50)	1.13^{c} (6.08)	1.18^{b} (6.17)
$B_5$5N.5B	5.0	0.5	-	-	31.49^{cd} (34.10)	35.82^{j} (36.74)	1.02^{c} (5.79)	0.98^{b} (5.62)
Mean					**20.05 (26.89)**	**76.22 (60.78)**	**3.67 (11.04)**	**2.33 (8.77)**
CD (0.05)					**3.32**	**4.00**	**3.72**	**3.09**

1 Figures in parenthesis are transformed values (Arc-sine transformation).

1 Values within column followed by different letters are significantly different at 5% probability level.

at par superior for shoot proliferating efficiency. Culture media B_5.5B.5N (9.32) and B_5.5Kn.5N (7.25) proved significantly superior for producing shootlets in higher numbers. Shoot with higher length were attained on culture media B_5.5B.5N (5.20 cm), B_5.5Kn.5N (4.79 cm) and B_5.1Td.5N (3.84 cm).

Shootlets of Liquorice were transferred on sixteen different fortifications of basal B_5 medium for induction of *in vitro* rooting (Table 4). Maximum *in vitro* root proliferating ability was demonstrated by B_5.5I (84.92 %), B_5I (83.27 %), B_5I.5B (82.92 %) and B_5I.5Kn (82.90 %) with statistically *at par* performance. For higher number(s) of root initiation per explant, rooting media B_5.5I (11.75) and B_5I (8.29) were found to be significantly superior. The roots of higher length was developed by B_5I (3.42 cm) closely followed by B_5.5I (2.13cm).

Table 4. Effects of different plant growth regulators on *in vitro* rooting of shootlets in liquorice.

Culture Media	Plant growth regulators (mgl^{-1})				*In vitro* rooting response		
	IBA	NAA	BA	Kn	Root proliferating shootlets (%)	No. of roots per shootlet	Mean root length (in cm)
B_5.5I	0.5	-	-	-	84.92^{a} (67.10)	11.75^{a} (19.97)	2.13^{ab} (8.34)
B_5I	1.0	-	-	-	83.27^{a} (65.83)	8.29^{ab} (16.71)	3.42^{a} (10.62)
$B_5$2I	2.0	-	-	-	55.97^{d} (48.41)	5.38^{c} (13.37)	1.19^{b} (6.24)
$B_5$3I	3.0	-	-	-	41.36^{e} (40.00)	3.12^{d} (10.12)	1.12^{b} (6.06)
B_5.5N	-	0.5	-	-	37.92^{f} (37.98))	2.64^{d} (9.30)	0.92^{b} (5.47)
B_5N	-	1.0	-	-	33.25^{g} (35.19)	3.84^{d} (11.19)	1.07^{b} (5.90)
$B_5$2N	-	2.0	-	-	32.75^{g} (34.89)	2.68^{d} (9.21)	0.62^{b} (4.45)
$B_5$3N	-	3.0	-	-	29.49^{h} (32.87	2.32^{d} (8.74)	0.78^{b} (5.03)
B_5.5Kn	-	-	-	0.5	59.28^{c} (50.32)	3.92^{cd} (11.30)	1.35^{b} (6.63)
B_5Kn	-	-	-	1.0	38.08^{f} (38.08)	3.18^{d} (10.06)	0.97^{b} (5.56)
$B_5$2Kn	-	-	-	2.0	32.19^{gh} (34.55)	2.68^{d} (9.37)	1.05^{b} (5.87)
$B_5$3Kn	-	-	-	3.0	29.36^{h} (32.78)	1.19^{d} (6.24)	1.03^{b} (5.80)
B_5 I.5B	1.0	-	0.5	-	82.92^{a} (65.82)	8.20^{b} (16.62)	2.12^{b} (8.36)
B52I.5B	2.0	-	0.5	-	80.02^{b} (63.43)	7.09^{bc} (15.43)	1.72^{b} (7.47)
B_5 I.5Kn	1.0	-		0.5	82.90^{ab} (65.58)	7.89^{b} (16.23)	1.98^{b} (8.01)
$B_5$2I.5Kn	2.0	-		0.5	79.85^{b} (63.32)	5.32^{c} (13.30)	1.64^{b} (7.31)
Mean					**55.22 (47.97)**	**4.96 (12.86)**	**1.44 (6.88)**
CD (0.05)					**3.06**	**3.69**	**2.05**

Figures in parenthesis are transformed values (Arc-sine transformation).
Values within column followed by different letters are significantly different at 5% probability level.

DISCUSSIONS

The goal of *in vitro* culture of liquorice is to develop reliable protocol for achieving plant regeneration in higher frequencies from nodal segment cultures in order to use them for mass clonal propagation of desirable genotypes or cultivars.

During present investigation basal B_5 medium was used throughout the experiment, as this was found more responsive as compared to MS in course of preliminary experiments. As per studies conducted so far, composition of culture media does not seem to play major role in *in vitro* response as much as the type and concentration of plant growth regulators. Culture medium fortified with higher concentrations of auxins in alone (2, 4-D, NAA or 2, 4, 5-T) initiated calli in higher frequencies. Higher degree of callus initiation was observed on culture media $B_5$2D, $B_5$3D and $B_5$3N as compared to culture media $B_5$3N.5Kn and $B_5$4N.5B containing higher concentration of an auxin in combination with lower concentration of cytokinins, $B_5$5B.5N and $B_5$5Kn.5N supplemented with relatively higher concentration of cytokinins in combination with lower concentration of an auxin as well as B_5B, B_5Kn, $B_5$2Td with cytokinins at higher concentration suggested that an auxin alone is adequate for higher degree of callus initiation from cultured nodal segments.

Regeneration of multiple shoots from nodal segment explants directly as well as from nodal segment-derived callus of liquorice has been reported earlier in response to cytokinins BA, Kn and TDZ (Patel and Shah 2007). Both adenine (BA and Kn) and phenyl urea derivatives

(TDZ) of cytokinins were used in the present study for shoot regeneration. It was observed that naturally occurring adenine derivatives were better for shoot multiplication when compared to TDZ. Growth regulator BAP promotes adventitious buds in excised organs and tissues *in vitro* (Bhojwani and Johri 1971). Present finding also revealed that BAP favoured adventitious multiple shoots. The regeneration of multiple adventitious shoot buds in different concentrations of BAP has also been observed in case of *Murraya koenigii* (Rajendra and D'Souza 1998) and *Peganum harmala* (Saini and Jaiswal 2000). BA was found superior for shoot proliferation of *Glycyrrhiza glabra* in the earlier studies (Patel and Shah 2007). During the present study, it was also observed that Kn and TDZ were equally effective for shoot regeneration but the number of shoots formed per explant were better in the presence of BA.

Kadota and Niimi (2003) reported BA more suitable for shoot proliferation for pears than synthetic phenyl urea derivatives. TDZ was found more effective at lower concentrations (0.1-0.2 mgl^{-1}) as compared to BA and Kn (they were found more effective at the concentration of 0.5mgl^{-1}). Effectiveness of TDZ at lower level perhaps, was due to induced accumulation of endogenous cytokinins, as reported earlier by Murthy *et al.* (1995). TDZ also affected auxin transport in hypocotyl tissues of *Pelargonium* and others (Murch and Saxena, 2001) and promoted regeneration frequency by altering the levels of abscisic acid, ethylene and perhaps proline (Murch and Saxena 2001). In the present study, TDZ was found to be unresponsive when supplemented into media at the concentration beyond to 0.2 mgl^{-1}. This finding is in accordance with the findings of Huetteman and Preece (1993) who also reported that TDZ at higher concentrations inhibit shoot elongation in many species. However, the exact mechanism of TDZ induced shoot proliferation in plants is not all that clear.

During the present study, medium supplemented with NAA in combination with cytokinins (BA, Kn or TDZ) has shown to promote shoot bud differentiation. These results are in agreement with Agastian *et al.* (2006) in *Justicia. gendarussa*, *Rauwolifia tetraphylla* (Ghosh and Banerajee 2003) and *Withania somnifera* (Kannan *et al.* 2005).

More responsive shoot proliferation efficacy of culture media B_5.5B.5N and B_5.5Kn.5N with equal quantity of auxin and cytokinin as compared to nutrient media $B_5$5N.5B with relatively a higher auxin and lower cytokinin or $B_5$5B.5N with relatively higher cytokinin with lower auxin suggested that culture medium was more effective when a cytokinin and an auxin were added in equal proportion. The quality of shoots and the overall growth response was better in these growth regulators combinations. A comparatively lower response was recorded when cytokinins was used separately in the medium. Much lower results were documented with supplementation of auxins alone into medium. A review of literatures indicates that addition of either 2,4-D or NAA in the culture medium improved the response in a number of species in terms of overall shoot growth. We observed that 0.5 mgl^{-1} BA solely in B_5 medium proliferated maximum shoots (86.28 %) higher shoots per explant (4.7) and higher length (3.13). On the other hand, addition of 0.5 mgl^{-1} NAA and BA each, elevated all the *in vitro* responses considerably. Maximum shoot proliferating ability (94.12 %), higher numbers of shoots per explant (9.32) and shoot of maximum length (5.20) have be achieved with the combined application of auxin and cytokinin. Much lower results were documented with application of 0.5 mgl^{-1} NAA (alone) in the medium. The Present study also pointed out the synergistic effect of both auxins (NAA) and cytokinin (BAP) favores profuse growth of multiple shoots. Recovery of shoots in higher numbers on medium supplemented with combination of an auxin with a cytokinin as compared to supplementation of an auxin as well as a cytokinin separately perhaps due to occurrence of direct as well as indirect organogenic mode of regeneration concurrently.

During present investigation, rhizogenesis frequencies were higher when shootlets were transferring to the rooting medium. In general, auxins like IBA, IAA as well as NAA were effective for inducing *in vitro* rooting. B_5 medium supplemented with 0.5-1.0 mgl^{-1} IBA was found to be optimum for inducing *in vitro rooting*. Maximum *in vitro* root proliferation, number of roots and root length were observed on rooting media B_5.5I followed by B_5I. The results clearly indicated that rooting of *in vitro* shoots of liquorice required lower to moderate concentrations of IBA responded better as compared to

culture media fortified with NAA, BA or Kn. Medium supplemented with higher auxins enhanced the intensity of callus induction, while, the number of roots produced per shoot and root growth reduced considerably. Similar response has also been observed by Behera *et al*. (2008) for cotyledonary node derived *in vitro* rooting response of *Gmelina arborea*. Auxins promoted adventitious root development on intact plants as well as excised stems. Among auxins, IBA was the most effective than any other synthetic auxins in the most of the cases apparently because it is not destroyed by IAA oxidase or other enzymes and therefore persists longer. These results are in conformity with the earlier findings of Patel and Shah (2007) for nodal segment culture of liquorice.

In conclusion, the present results substantiate the fact that nodal segments is an excellent explant source and are available throughout the year especially in liquorice where choice of explants is limited due to non-availability of seeds. The present study shows that nodal segment explants of liquorice carry a high potential for rapid multiple shoot regeneration and subsequent micropropagation. This protocol provides a successful and rapid technique that can be used for mass *in vitro* propagation of elite species. Furthermore, these techniques may be used for raising embryogenic cell suspension cultures for production of secondary metabolite products for exploiting these in pharmaceutical industries.

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