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Micropropagation and Plant Regeneration of *Rauvolfia serpentina* by Tissue Culture Technique

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Abstract: The shoots of *Rauvolfia serpentina* from the field growing plants were used as explants and cultured on MS medium supplemented with 2.5 mg/L BA + 0.1 mg/L NAA to afford multiple shoots. It was evident that MS + 0.5 mg/L BA + 0.2 mg/L NAA was the most suitable combination for induction of callus and development of shoot buds formation but it failed to support elongation of shoot. So, the cultures with tiny shoots was transferred to 2.0 mg/L BA with 0.05mg/L NAA, where they showed excellent elongation. Regenerated shoots rooted well 0.2 mg/L IBA+ 0.2mg/L NAA combination. After having acclimatization the regenerated plantlets were transferred to the open field where 95% of the plant-lets survived and showed excellent growth.

Key words: *Rauvolfia serpentina,* medicinal plant, tissue culture

Introduction

Rauvolfia serpentina (L.) Benth is a perennial herb belonging to the family Apocynaceae. Its roots are used as a valuable remedy for high blood pressure, insomnia, anxiety, excitement, schizophrenia, insanity, epilepsy, hypochondria, and other disorders of the central nervous system (Kirtikar and Busu, 1993). The inhabitants of Macassar use the petioles as an antidote for Ipoh. Infusion, decoction, and extract of the roots are employed to increase uterine contractions for expulsion of fetus, to treat painful affections of bowels, diarrhea, dysentery, cholera, and colic (Ghani, 1998). This important medicinal plant holds the tremendous potentialities for massive propagation towards the commercialization of the alkaloid reserpine.

Propagation of *Rauvolfia serpentina* through tissue culture has previously been described but most of them have focused on micropropagation by means of stimulating axillary shoot growth (Mathur *et al.,* 1987; 1993; Roy *et al.,* 1994). Sarkar *et al.* (1996) also reported induction of callus and plant regeneration from callus cultures in *Rauvolfia serpentina* on MS medium. But the number of axillary shoot growth and the number of regenerated plantlets were not so high. The naturally or conventionally this plant does not get adequate opportunity to propagate on large scale. Because uprooting of this valuable plant used by the local Ayurvedic, Unani practitioners to remedy different kinds of diseases for centuries together. It is also noted that their process of collection is from wild natural habitat.

For this reason the plant producing alkaloids widely, has become very rare in Bangladesh. For the conservation of this genotype we established a protocol for its mass propagation. In this experiment, shoot tip and node from the field grown plants were used as primary explants for axillary shoot proliferation. Subsequently (from aseptically raised axillary shoots) internode and leaf were used for induction of callus and regeneration from that callus culture.

Materials and Methods

The whole research project was completed in the Department of Botany, University of Rajshahi, Bangladesh during April 1999 – September 2000.

Aseptic culture: *Rauvolfia serpentina* grown at the medicinal plant garden of the Pharmacy Department of the Rajshahi University Campus was used as the explant source. Shoot tips, collected from 3-4 years old plants were washed in running tap water for 25 minutes followed by DW containing 1% savlon (v/v) for 10 minutes. The shoots having tips and node portions with axillary buds were then washed repeatedly with distilled water and finally treated with $HgCl_2$ (0.1%) for 6 min in a laminar flow cabinet and washed four times with autoclaved double distilled water to remove any trace of $HgCl_2$ solution. Subsequently internode and leaf explants were taken from 5-6 weeks old aseptically grown shoots. The explants were cultured on MS medium supplemented with different concentration and combinations of cytokinine and auxins for inducing callus derived shoot regeneration. The basal medium used for all the experiments were Murashige and Skoog mineral formulation (MS), containing standard salts and vitamins, 30 mg/l sucrose and 6 mg/l (unless otherwise stated) plant tissue culture grade agar. The media were variously supplemented with 6-benzyl adenine (BA), 6-furfuryl amino purine (Kinetin, Kn) either individually or in different combinations with auxins, α-napthalene acetic acid (NAA), Indole 3-butyric acid (IBA), Indole 3-acetic acid (IAA). The pH was adjusted to 5.7 (by digital pH meter) before adding agar, and the media were autoclaved at 1.1 kg/cm for 20 min. at 120 °C. Cultures were incubated at 25 ± 1°C with a photoperiod of 16 hrs at 3000 lux light intensity of cool white fluorescent light.

All cultures were initiated in 150 x 25 mm^2 glass tube containing 15-20 ml of medium. The cultures were regularly subcultured on fresh medium at 4 weeks intervals in glass tubes or 100 ml flasks. Observations were recorded every 5 days following inoculation and subculturing. All experiments were repeated twice with at least 15 cultures per treatment. Subsequently different types of explants shoot tip, node, internode and leaf segments were then prepared from *in vitro* raised cultures and were used as secondary explants to conduct different experiments in the on going investigation.

Multiplication of shoots: For inducing adventitious shoots and their development, internode and leaf explants were cultured on MS medium supplemented with different concentrations and combinations of cytokinin and auxins. It was evident that 0.5 ml/L BA + 0.2 ml/L NAA was the most suitable combination for induction callus and shoot bud formation but failed to show elongation. But, when the explant shoot bud forming stage was transferred to 2.0 ml/L BA with comparatively very low concentration to auxin 0.05 mg/L NAA, they showed excellent elongation.

Rooting: Proliferated shoot of 2-4 cm length were rescued aseptically from the culture vessels and cultured on freshly

prepared half strength MS macro and micro-nutrients contained in glass tubes. The media were variously supplemented with NAA and IBA in different concentrations for root induction.

Transplantation: Rooted plantlets were kept in a room of normal temperature (30 ± 2°C) at normally daylight for 7 days. Plantlets were taken out from the culture tubes and washed carefully under running tap water for complete removal of the media. The *in vitro* derived plant acclimated very fine when they were transferred to plastic tray containing "autoclaved coco-peat" as potting mixture then that of garden soil, compost and sand in a ratio 2:2:1. The trays were kept in a shady place, covered with transparent box like polyethylene tent (for the cause of maintaining humidity) and were watered one time daily. After 3-4 weeks when the plants were fully acclimated to outdoor conditions they were again transplanted individually to bigger pots containing compost and soil (1:1).

The pants were watered periodically and upper layer of the soil was nuclide occasionally whenever necessary for better aeration.

Results and Discussion

Generally multiple shoot initiated to grow within two weeks and afterwards some cultures were found to grow base callus around their basal portion. The mean values for shoot, average length of shoot and base callus observed after 5 weeks are presented in Table 1. In the present study when cytokinin was used alone (without combination of auxin), it was observed that BA was more effective for shoot multiplication than Kin. On the other hand, with combinations of BA, Kin, IBA and NAA, 2.5 mg/l BA + 0.1 mg/l NAA (lower amount of auxin) was found to be the best combination for shoot multiplication and proliferation. These results are significant for field grown (shoot tip, nodal) explants and also *in vitro* grown explants.

So, addition of exogenous auxins to the medium promoted axillary shoot proliferation from nodes and shoot tips of explants and enhanced the growth of culture. One of the advantages of adding auxin at low concentration on the culture media is to nullify the effect of the higher concentrations of cytokinin on axillary shoot elongation (Hu and Wang, 1983). In the present experiment on shoot tip and nodal explants an average more than 5 shoots were produced on media having 2.5 mg/l BA + 0.1 mg/l NAA and 93.33% of shoots were proliferated and maximum average number of shoots per explant was 5.9 ± 0.63 . Some of the shoots grew rapidly and others grew slowly (Fig. 1A). In this study higher amounts of cytokinin and auxin or comparatively higher amount of auxin with the cytokinin and auxin combination could not produce higher number of shoot proliferation but in this condition produced moderate and massive callus at the basal portion of the shoots of explants and which greatly inhibited (Table 1) the growth of multiple shoots in culture.

Thus, for inducing the adventitious shoots and their development, internodes and leaf explants were (taken from *in vitro* proliferated shoots) cultured on MS medium observed and average highest length of shoot (4.1± 0.55cm) was found at the same combination (Fig. 1B).

Among the BA-IBA formulations maximum frequency of 75% explants showed shoot bud elongation at 2.0mg/L BA with 0.05mg/L IBA. This combination also produced 4.9± 0.78 shoots per culture. On the other hand BA-IAA combinations supplemented with different concentrations and combinations of cytokinin and auxin. Among this MS + 0.5 mg/L BA + 0.2

Table 1: Effect of different concentrations and combinations of growth regulator in MS media on morphogenic response of shoot explant on *Rauvolfia serpentina*.

Growth regulators (mg/L)	% of shoot formation	Average number of shoots per culture	Average length of shoots (cm)	Base callusing
BA				
0.5	40.00	2.6± 0.27	3.5± 0.43	+
1.0	46.66	3.3± 0.15	3.9± 0.20	+
2.0	60.0	3.9± 0.012	3.9± 0.18	+
2.5	73.33	4.4± 0.39	4.2± 0.26	+
3.0	46.66	3.2± 0.41	3.3± 0.39	+ +
3.5	33.33	2.1± 0.30	3.0± 0.32	+ +
Kin				
0.5	33.33	2.0± 0.22	1.8± 0.52	+
1.0	40.00	2.6± 0.31	2.9± 0.33	+
2.0	46.66	3.0± 0.40	2.0± 0.18	+
2.5	60.00	3.8± 0.24	2.8± 0.27	+
3.0	40.00	2.9± 0.15	2.6± 0.22	+
3.5	26.66	2.1± 0.21	2.0± 0.31	+ +
BA +NAA				
2.0+ 0.1	80.00	4.3± 0.42	3.2± 0.51	+
2.0+ 0.2	40.00	2.2± 0.17	2.0± 0.41	+ +
2.0+ 0.5	-	-	-	+ + +
2.5+ 0.1	93.33	5.9± 0.63	3.9± 0.71	+ +
2.5+ 0.2	53.33	3.4± 0.59	2.6± 0.37	+ +
2.5+ 0.5	-	-	-	+ + +
BA +IBA				
2.0+ 0.1	73.33	3.6± 0.64	2.7± 0.57	+
2.0+ 0.2	26.66	2.0± 0.29	2.0± 0.42	+ + +
2.0+ 0.5	-	-	-	+ + +
2.5+ 0.1	86.66	4.6± 0.32	3.1± 0.44	+
2.5+ 0.2	33.33	2.9± 0.43	2.1± 0.47	+ +
2.5+ 0.5	-	-	-	+ + +
Kin +NAA				
2.0+ 0.1	73.33	3.9± 0.32	2.8± 0.73	+
2.0+ 0.2	40.00	2.0± 0.23	2.0± 0.32	+ + +
2.0+ 0.5	-	-	-	+ + +
2.5+ 0.1	86.66	4.6± 0.71	3.2± 0.72	+
2.5+ 0.2	46.66	3.0± 0.29	2.1± 0.17	+ +
2.5+ 0.5	-	-	-	+ + +
Kin +IBA				
2.0+ 0.1	66.66	3.2± 0.55	2.3± 0.69	+
2.0+ 0.2	20.00	1.5± 0.61	1.0± 0.25	+ + +
2.0+ 0.5	-	-	-	+ + +
2.5+ 0.1	80.00	4.1± 0.83	2.6± 0.42	+ +
2.5+ 0.2	26.00	2.6± 0.22	1.8± 0.44	+ + +
2.5+ 0.5	-	-	-	+ + +

(-) no response; (+) slight callusing; (+ +) considerable callusing and (+ + +) profuse callusing.

Each treatment was comprised of 15 explants and data (X ± S.E) were recorded after 35 days of culture.

mg/L NAA and it was the most suitable combination. At that combination, cultured explants showed best callus induction and shoot bud formation (90%) but they failed to show elongation.

Moreover, when internode derived calli with tiny shoot bud forming stage were transferred to 2.0mg/L BA with comparatively low concentration to auxin (0.05 mg/l), they showed excellent elongation. The mean values for shoot, average length of shoots , maximum number of shoots were collected after 12 weeks (Table 2). Among various combinations of BA and NAA the cultured explants showed the best results on the medium MS with 2.0mg/L BA + 0.05mg/L NAA. This combination also showed maximum percentage (81.25%) of adventitious shoot proliferation and maximum number of 6.4± 0.74 shoots per culture was maximum frequency of 68.75% explants showed shoot buds differentiation at 2.5mg/L BA with 0.5mg/L IAA. Maximum

Table 2: Effects of different concentrations and combinations of BA and Auxin on regeneration of adventitious shoot from internode derived callus.

Growth regulators type and concentration (gm/L)	% of shoot formation	Number of total shoot per culture	Number of usable shoot per culture	Average length of shoot per culture (cm)
BA				
1.0	-	-	-	-
1.5	-	-	-	-
2.0	-	-	-	-
BA +NAA				
1.0+ 0.05	25.00	2.1± 0.21	1.8± 0.29	2.1± 0.31
+ 0.1	-	-	-	-
+ 0.2	-	-	-	-
1.5+ 0.05	56.25	4.1± 0.52	3.5± 0.28	2.7± 0.26
+ 0.1	37.50	3.6± 0.43	2.7± 0.22	2.8± 0.37
+ 0.2	-	-	-	-
2.0+ 0.05	81.25	6.4± 0.74	4.9± 0.68	4.1± 0.55
+ 0.1	65.50	4.9± 0.51	4.4± 0.61	3.7± 0.45
+ 0.2	25.00	3.2± 0.60	2.8± 0.57	2.5± 0.26
BA +IBA				
1.0+ 0.05	18.75	1.8± 0.58	1.3± 0.71	2.0± 0.51
+ 0.1	-	-	-	-
+ 0.2	-	-	-	-
1.5+ 0.05	50.00	3.8± 0.33	3.3± 0.70	2.5± 0.23
+ 0.1	31.25	3.4± 0.52	2.5± 0.56	2.3± 0.28
+ 0.2	-	-	-	-
2.0+ 0.05	75.00	4.9± 0.78	4.4± 0.61	30.42.6±
+ 0.1	56.25	4.3± 0.71	4.0± 0.63	3.3± 0.54
+ 0.2	18.75	3.0± 0.51	2.6± 0.42	2.3± 0.4
BA +IAA				
1.0+ 0.05	12.50	1.5± 0.34	-	-
+ 0.1	-	-	-	-
+ 0.2	-	-	-	-
1.5+ 0.05	43.75	3.4± 0.35	3.1± 0.55	2.3± 0.42
+ 0.1	25.00	2.8± 0.22	2.3± 0.71	2.1± 0.18
+ 0.2	-	-	-	-
2.0+ 0.05	68.75	4.4± 0.62	3.9± 0.28	3.2± 0.66
+ 0.1	50.00	3.8± 0.76	3.4± 0.66	2.8± 0.27
+ 0.2	12.50	2.7± 0.34	2.1± 0.47	2.0± 0.25

(-) Indicate no response. There were 16 explants in each treatment and data (X ± S.E) were collected after 12 weeks.

Table 3: Effects of different concentrations and combinations of BA and Auxin on regeneration of adventitious shoot from leaf derived callus.

Growth regulators type and concentration (gm/L)	% of shoot formation	Number of total shoots per culture	Number of usable shoots per culture	Average length of shoots per culture (cm)
BA				
1.0	-	-	-	-
1.5	-	-	-	-
2.0	-	-	-	-
BA +NAA				
1.0+ 0.05	27.77	2.9± 0.23	2.0± 0.19	2.4± 0.42
+ 0.1	22.22	1.7± 0.29	-	-
+ 0.2	-	-	-	-
1.5+ 0.05	55.55	5.6± 0.57	4.1± 0.39	2.8± 0.31
+ 0.1	38.88	4.1± 0.48	3.0± 0.45	2.9± 0.58
+ 0.2	11.11	2.2± 0.24	1.9± 0.15	1.8± 0.22
2.0+ 0.05	94.44	8.7± 0.79	6.3± 0.72	4.6± 0.52
+ 0.1	66.66	5.4± 0.63	4.6± 0.43	3.9± 0.27
+ 0.2	31.25	3.8± 0.34	3.1± 0.49	2.8± 0.36
BA +IBA				
1.0+ 0.05	22.22	2.3± 0.21	1.8± 0.22	1.6± 0.31
+ 0.1	16.66	1.2± 0.24	-	-
+ 0.2	-	-	-	-
1.5+ 0.05	50.00	4.3± 0.51	3.8± 0.32	2.3± 0.23
+ 0.1	33.33	3.7± 0.32	2.7± 0.36	2.4± 0.35
+ 0.2	-	-	-	-
2.0+ 0.05	77.77	5.1± 0.44	4.1± 0.28	3.8± 0.34
+ 0.1	61.11	4.2± 0.32	3.8± 0.27	3.1± 0.25
+ 0.2	27.77	3.2± 0.47	2.7± 0.31	2.4± 0.20
BA +IAA				
1.0+ 0.05	11.11	1.7± 0.34	1.3± 0.16	1.2± 0.14
+ 0.1	-	-	-	-
+ 0.2	-	-	-	-
1.5+ 0.05	44.44	3.7± 0.33	3.2± 0.42	2.0± 0.23
+ 0.1	27.77	3.0± 0.21	2.2± 0.20	1.6± 0.17
+ 0.2	-	-	-	-
2.0+ 0.05	72.22	4.5± 0.52	3.8± 0.37	3.2± 0.55
+ 0.1	55.55	4.0± 0.41	3.3± 0.38	2.7± 0.25
+ 0.2	16.66	2.9± 0.32	2.4± 0.24	2.1± 0.30

(-) Indicate no response. There were 18 explants in each treatment and data (X ± S.E) were collected after 16 weeks

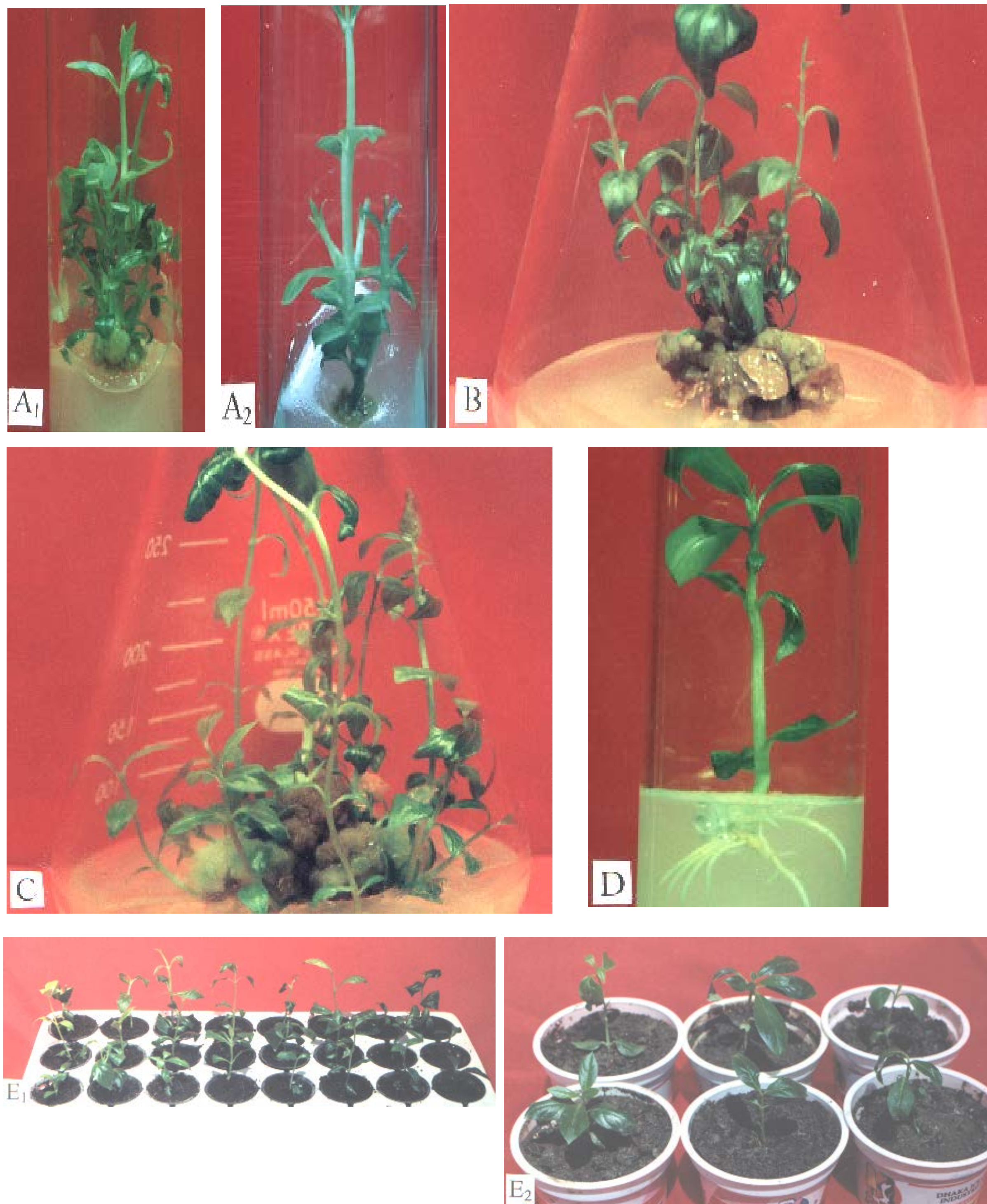

Fig. 1: Regeneration of plantlets in *Rauvolfia serpentina*.
A: Developments of shoots from A_1 shoot tip and A_2 nodal explants after 4 weeks of culture.
B: Development of adventitious shoots from callus of inter-node explants after 9 weeks.
C: Development of adventitious shoots from callus of leaf explants after 10 weeks.
D: Development of adventitious roots on regenerated shoots.
E: Regenerated plantlets established on 'coco-peat' after 3 weeks, E_1 and on soil after 7 weeks E_2, under *in vitro* condition.

Table 4: Effects of auxin in half strength MS medium with 3% sucrose on adventitious root formation *in vitro* from *R. serpentina* micro-cuttings cultured for 5 weeks at 25°C under 16 hour photo period

Hormonal supplement (mg/L)	% of cutting rooted	Number of roots per rooted cutting	Average length of the root (cm)	Days to root formation	Callus formation at the cutting base
Ho	-	-	-	-	-
NAA					
0.1	-	-	-	-	+ +
0.2	13.33	1.1± 0.19	1.0± 0.52	30-35	+ + +
0.5	-	-	-	-	+ + +
IAA					
0.1	20.00	2.8± 0.32	1.2± 0.19	30-32	+
0.2	26.66	3.0± 0.57	1.4± 0.24	28-30	+ + +
0.5	13.33	2.1± 0.51	1.7± 0.22	30-30	+ + +
IBA					
0.1	33.33	4.2± 0.45	2.1± 0.34	26-28	+
0.2	53.33	5.3± 0.58	2.0± 0.28	24-26	+
0.5	40.00	5.1± 0.48	2.0± 0.22	24-26	+ + +
IBA +NAA					
0.1+ 0.1	66.66	6.2± 0.69	2.2± 0.33	18-20	+ +
0.2+ 0.1	73.33	6.7± 0.77	2.2± 0.38	18-20	+ +
0.2+ 0.2	100.00	7.3± 0.83	2.5± 0.52	14-16	+
0.5+ 0.5	33.33	3.6± 0.27	2.2± 0.33	18-22	+ + +
IBA +IAA					
0.1+ 0.1	40.00	5.6± 0.31	2.3± 0.23	18-24	+
0.2+ 0.1	60.00	5.8± 0.41	2.0± 0.32	18-22	+ +
0.2+ 0.2	66.00	6.1± 0.44	2.2± 0.44	20-24	+ +
0.5+ 0.5	26.66	3.2± 0.39	1.6± 0.52	22-24	+ + +

H_0 = Hormone free (-) Indicate no response; (+) slight callusing; (+ +) considerable callusing and (+ + +) profuse callusing.
There were 15 explants in each treatment and data (X ± S.E) were collected after 5 weeks

number of shoot (4.4± 0.62) per culture was recorded at the same combination. Among different growth regulators BA alone and 1.0mg/L BA with 0.1 - 0.2mg/L NAA, IBA or IAA did not produce any shoot bud (Table 2). They produced only callus from the explants. This callus produced shoots when it was sub-cultured on MS medium supplemented with 2.0mg/L BA + 0.05mg/L NAA.
Leaf explants were taken from *in vitro* proliferated shoots and were cultured on MS medium supplemented with different concentrations and combinations of cytokinin and auxin for induction callus derived shoots. Data were collected after 16 weeks (Table 3). Among this, BA (1.0, 1.5 and 2.0mg/L) alone and in combination with different auxins viz. NAA, IBA and IAA. Under appropriate combinations of cytokinin and auxin the leaf explants showed formation of a number of adventitious shoot buds within 7-9 weeks of culture. BA alone did not produce any shoot bud and it also failed to produce considerable amount of callus. Among BA-NAA combinations maximum frequency of 94.44% explants showed shoot bud differentiation at 2.0 mg/L BA with 0.05mg/L NAA. Number of shoot proliferation was maximum (8.7± 0.79) at 2.0mg/L BA with 0.05mg/L NAA (Table 3, Fig. 1C). Among different growth regulator supplements BA alone and combinations 1.0mg/L BA with 0.2mg/L NAA, 1 mg/L BA + 0.2mg/L IBA, 1.5mg/L BA + 0.2mg/L IBA, 1.0mg/L BA + 0.1-0.2mg/L IAA, 1.5mg/L BA + 0.2mg/L IAA had inhibitory effects on organogenesis (Table 3). However, they produced only fast growing callus. When this callus was subcultured on MS medium supplemented with 2.0mg/L BA with 0.05mg/L NAA high percentage of shoot bud differentiation was observed.

Root induction: Proliferated shoots were excised from the culture tubes and cultured on ½ MS (MS with ½ strength of major salts only) medium containing different concentrations and combinations of NAA, IAA and IBA. The percentage of root formation, number of roots per shoot and length of the longest root were recorded after 4-5 weeks of culture. The rooting response to different auxin treatments is shown in Table 4. No rooting was found at auxin free medium. Percentage of root induction and number of roots per shoot were noticeably influenced by the combination, concentration, and type of auxin. Among three types of auxin IBA was found to be comparatively more effective than other two auxins NAA and IAA at different concentrations tested for producing roots. On the other hand, among the auxin 0.2 mg/L IBA + 0.2 mg/L NAA combination was found to be the best combination of auxins for proper rooting in which 100% shoots rooted within 4-5 weeks of culture and the highest average number of roots was found to be 6.0 (Table 4, Fig. 1D) The *In vitro* grown plants were transplanted to the pots and 95% of plants were survived (Fig. 1E).
Through the present study protocols for routine plantlet regeneration from the shoot and nodal explants of field grown plants and from calli of leaf and internode explants of *in vitro* grown shoots have been established. Manipulation of cytokinin (BA) and auxin (NAA) concentrations was found to be very important for effective regeneration of complete plants that can be used either for mass-scale propagation of elite genotype or selection of somaclones from callus derived plants with higher alkaloid concentration. The protocols described here are reproducible and may be exploited for the above mentioned purposes.

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