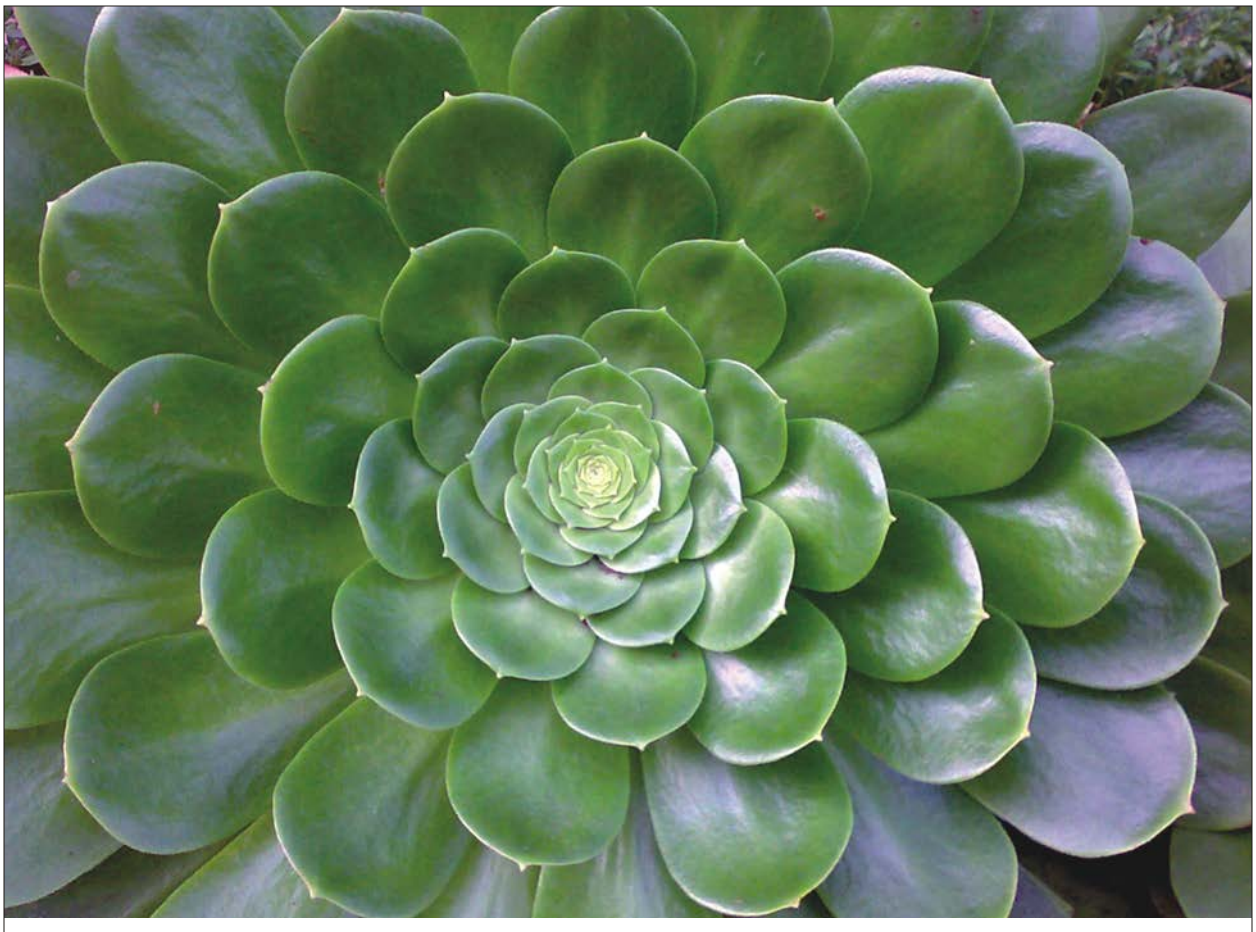

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Protocol Establishment for Multiplication and Regeneration of *Ocimum sanctum* Linn. An Important Medicinal Plant with High Religious Value in Bangladesh

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Abstract: The experiment was conducted to Plant Biotechnology Laboratory of Institute of Biological Sciences, Rajshahi, Bangladesh. Shoot tip and leaf explants of *Ocimum sanctum* Linn. were cultured on different concentrations and combinations of growth regulators (BAP, Kin, 2, 4-D, IAA and IBA) in MS medium to observe shoot multiplication, callus induction, callus regeneration and root induction. Among the different concentrations and combinations of growth regulators, the highest percentage of shoot formation and highest average number of shoots were observed 90 and 5.88%, respectively in 0.2 mg L^{-1} BAP from shoot tip explants. Callus induction was obtained within 12-15 days of culture from leaf explants. The highest frequency (90.00%) of organic callus induction was observed in MS medium containing 1.0 mg L^{-1} NAA. Shoot regeneration occurred when the calli were sub cultured in Ms medium supplemented in BAP formulation. The highest percentage of shoot regeneration was obtained 90.00 in 0.2 mg L^{-1} BAP. *In vitro* grown shoots rooted best on MS medium containing 0.1 mg L^{-1} NAA. The *in vitro* grown plant lets were transferred to pots containing sand and soil mixture, acclimatized in a culture room and finally rooted plants were transferred to soil.

Key words: *In vitro*, regeneration, multiplication, shoot tip, callus, *Ocimum sanctum*

INTRODUCTION

Ocimum sanctum Linn. belongs to the natural family Labiatae. The plant is an evergreen, monoecious, perennial medicinal herb with a height of 30-60 cm. It is an important medicinal plant in Bangladesh and used as herb against a number of disease leucoderma, strangury, lumbago, kapha and vata asthma, bronchitis, vomiting (Ghani, 2003). The leaves have expectorant properties and their juice is used catarrh and bronchitis. The preparation, also, is applied to the skin ringworm and their coetaneous disease. An infusion of the leaves is used as a stomachic in the gastric disorders of the children and in hepatic affections. The root is given in decoction as a diaphoretic in malarial fever. The bruised fresh roots stem and leaves are applied to the bites of mosquitoes (Kirtikar and Basu, 1994). This plant is presently grown as a homestead plant in almost all-tropical countries. It has another magnificent religion status in the Hindu community of the country and people used to worship to the plant growing and nourishing around homestead at least one plant in a holly place of each house (Hooker, 1885).

At the advent of plant biotechnology, like some other medicinal plants efforts have been made for micro propagation of this plant in artificial culture medium. Begum *et al*. (1999) obtained multiple shoot from node and shoot tip explants of *O. sanctum*. Regeneration of multiple shoot was also

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reported by Banu *et al*. (2001) from nodal explants of *O. sanctum*. Banu *et al*. (1999) also observed callus from shoot tip and nodal explants of *O. sanctum* on MS medium supplemented with BAP + NAA formulation. Available previous reports were on some specific aspects of regeneration and that necessitate to develop complete information of regeneration of the plant. The objective of their study was to establish a complete *in vitro* regeneration protocol for the plant *Ocimum sanctum* using all possible explants for direct regeneration, callus culture and callus regeneration to plantlet. Callus and cell culture of this important plant would generate the commercial possibility for isolation of secondary metabolites from its callus and cell culture.

MATERIALS AND METHODS

Shoot tip and leaf explants (1-2 cm) of *Ocimum sanctum* Linn. (Tulsi) were collected from mature plants used for establishing shoot formation and callus induction. The explants were washed thoroughly under tap water. Then treated with a few drops of Tween-80 and 1% savlon for 20 min. with constant shaking washed thoroughly with distilled water to remove gummy substant and savlon. Explants were surface sterilized with 0.1% $HgCl_2$ for 6 min followed by gentle shaking in a laminar air flow cabinet. After surface sterilization the explants were thoroughly washed for several times with sterile distilled water. Shoot tip explants then cut into pieces of 0.7-1.0 cm and transferred to 25×150 mm culture tubes with 15 mL agar-gelled MS (Murashige and Skoog, 1962) basal medium supplemented with different concentrations of BAP, Kin and combination with NAA for shoot initiation. The leaf explants cut into pieces of 1 cm and cultured on agar-gelled MS medium supplemented with different concentrations of 2,4-D and NAA for inducing callus. *In vitro* grown leaves were also cultured on MS medium containing same hormone. All media were supplemented with 30% sugar, adjusted to pH 5.8, gelled with 0.6% agar (BDH) and autoclaved at 120°C for 20 min under 1.1 kg cm^{-1} pressure. Cultures were incubated at 25±2°C under the warm fluorescent light with intensity varied from 2000-3000 lux. Similar sterilization techniques were reported by Evans *et al*. (1983), Thorpe (1981), Vasil (1984) and Pierik (1987). The calli was then transferred on MS medium containing different concentrations of BAP for differentiating shoots and incubated in light. Proliferated shoots were transferred to MS with different concentrations of NAA, IAA and IBA for root formation. Data were recorded after 28 days of culture.

RESULTS AND DISCUSSION

Direct Regeneration

For direct regeneration shoot tip of *Ocimum sanctum* was used as potented explant and it was cultured on MS medium supplemented with growth regulators BAP, Kin and NAA. Six concentrations (0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 mg L^{-1}) of BAP, seven concentrations (0.1, 0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 mg L^{-1}) of Kin were used in the investigation singly and in combination with four concentrations (0.1, 0.2, 0.5 and 1.0 mg L^{-1}) of NAA. Data were recorded on percentage of shoot formation, average number of shoot per explant, callus initiation and average length of shoot after 28 days of culture and their result are presented in Table 1. From the Table it is evident that BAP proved to be the most efficient in direct shoot regeneration of *O. sanctum* and the highest percentage was obtained in 0.2 mg L^{-1} BAP where 90% response was recorded in the culture. Under this concentration of BAP average number of shoot per explant was 5.88 and average length of shoot was 3.39 cm (Fig. 1A). Begum *et al*. (2002) also reported that 0.2 mg L^{-1} BAP was the most effective for shoot proliferation in *Ocimum basilicum* L. Sitakanta and Chand (1996) was also observed that the optimum BAP concentration for shoot proliferation was 0.25 mg L^{-1} for *Ocimum* sp. Superiority of BAP in producing *in vitro* shoots has also been confirmer in other plants like *Dianthus caryophyllus* (Sooch *et al*., 1998), *Melia azadarch* (Ahmed *et al*., 1993), *Dendrobium* (Lakshmidevi and Basu, 1991;

Table 1: Effect of different concentrations and combinations of BAP and NAA for shoot multiplication from shoot tip explants. Scoring was done after 28 days of culture and each treatment consists of 10 explants

Growth regulators (mg L^{-1})	Percentage of shoot formation	Average No. of shoot per explant	Callus initiation	Average length of shoot (in cm)
BAP				
0.1	80.00	3.62	-	2.68
0.2	90.00	5.88	-	3.39
0.5	80.00	4.87	-	3.06
1.0	60.00	2.50	-	2.17
2.0	50.00	2.00	+	1.60
5.0	50.00	1.80	++	0.30
Kin				
0.1	50.00	1.60	-	1.30
0.2	70.00	2.14	-	2.14
0.5	50.00	1.80	-	1.60
1.0	50.00	1.40	-	1.50
2.0	30.00	1.33	-	1.50
3.0	40.00	1.25	-	1.16
4.0	30.00	1.00	-	0.83
BAP + **NAA**				
0.2 + 0.1	80.00	2.12	+	1.50
0.2 + 0.2	60.00	1.84	+	1.33
0.2 + 0.5	50.00	1.60	+	1.20
0.2 + 1.0	40.00	1.25	++	1.00
0.5 + 0.1	60.00	1.67	+	1.17
0.5 + 0.2	50.00	1.60	+	1.10
0.5 + 0.5	40.00	1.25	+	1.00
0.5 + 1.0	40.00	1.25	++	1.00
1.0 + 0.1	60.00	1.34	++	1.17
1.0 + 0.2	50.00	1.20	++	1.00
1.0 + 0.5	30.00	1.00	++	0.84
1.0 + 1.0	20.00	1.00	++	0.57
Kin + **NAA**				
0.2 + 0.1	70.00	1.71	-	1.50
0.2 + 0.2	50.00	1.60	-	1.50
0.2 + 0.5	50.00	1.40	+	1.40
0.2 + 1.0	40.00	1.25	+	1.12
0.5 + 0.1	50.00	1.40	-	1.30
0.5 + 0.2	40.00	1.25	-	1.25
0.5 + 0.5	30.00	1.00	+	1.16
0.5 + 1.0	30.00	1.00	++	0.83
1.0 + 0.1	40.00	1.50	-	1.00
1.0 + 0.2	30.00	1.33	+	0.83
1.0 + 0.5	20.00	1.00	+	0.75
1.0 + 1.0	20.00	1.00	++	0.75

+ = Poor, ++ = Moderate, - = No callus formation

Honmode, 1992), *Strawberry* (Khan *et al*., 1998), *Ocimum sanctum* (Singh and Schgal, 1999). It has been noticed that with the increase of BAP the performance efficiency was found decreased. Under the treatment of BAP it is remarkable to note with the advancement of shoot initiation callus also found to induce around the explant culture on the medium.

Among the all concentrations of Kin the highest percentage of shoot was formation 70% in 0.2 mg L^{-1} Kin. In this concentration average number of shoot per explant was 2.14 and average length of shoot per culture was 2.14 cm (Table 1).

When the explant was cultured on BAP with NAA combination-then multiple shoot was obtained with callus. The highest frequency of multiple shoot was obtain in 0.2 mg L^{-1} BAP + 0.1 mg L^{-1} NAA where 80% of shoot formation and average number of shoot per explants were 2.12 recorded. Increase of BAP level and BAP, NAA combination in the medium the shoot tip explants produced callus with shooting (Table 1). The results are agreement with Banu *et al*. (2001) in *Ocimum sanctum*.

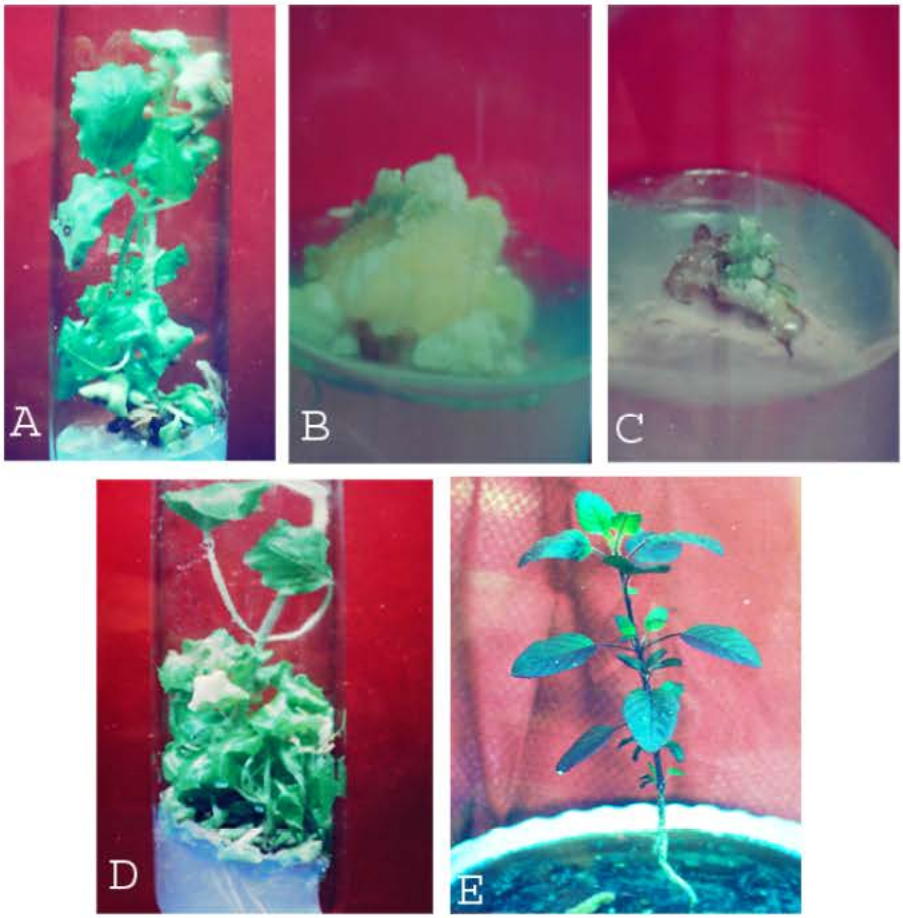

Fig. 1: (A) Multiple shoot formation from the shoot tip explant on MS medium with 0.2 mg L^{-1} BAP. (B) Induction of Callus on leaf explant on MS medium supplemented with 1.0 mg L^{-1} NAA after six weeks of culture. (C) Development of adventitious shoots on leaf derived callus after five weeks of culture. (D) Development of roots on MS medium with 0.1 mg L^{-1} NAA after eight weeks of culture and (E) Growth regenerated plantlets on the soil after 2 months transfer under *in vivo* condition

In all concentrations of Kin and NAA combination the highest percentage of shoot formation was 70%, highest average number of shoot per culture was 1.71 and highest average length of shoot was 1.50 cm in 0.2 mg L^{-1} Kin + 0.1 mg L^{-1} NAA. In the present experiment, the highest percentage of shoot induction was 68.33% in BAP under different plant growth regulators concentrations and combinations were used (Fig. 2). Average number of shoots per culture (3.44) and average length of shoots per culture 2.20 cm were also high in BAP than overall concentration and combination were used (Fig. 3 and 4).

Callus Induction

The leaf explants were cultured on MS medium supplemented with different concentrations (0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 mg L^{-1}) NAA and (0.1, 0.5, 1.0, 2.0, 3.0 and 5.0 mg L^{-1}) 2,4-D alone for callus induction (Table 2). Within 12-15 days the explants produced calli in NAA combination.

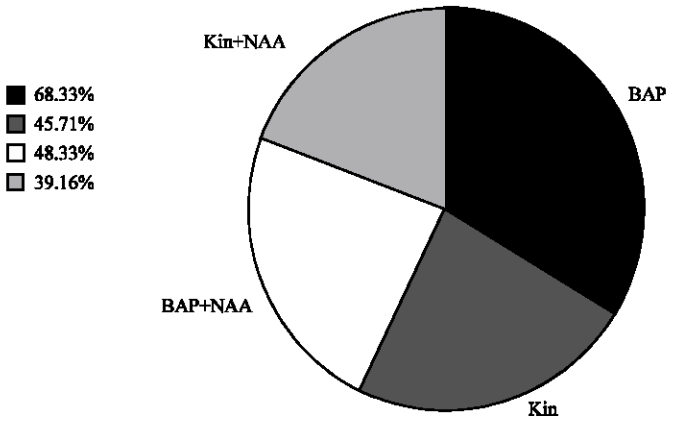

Fig. 2: Percentage of shoot induction under different plant growth regulators (overall concentration and combination)

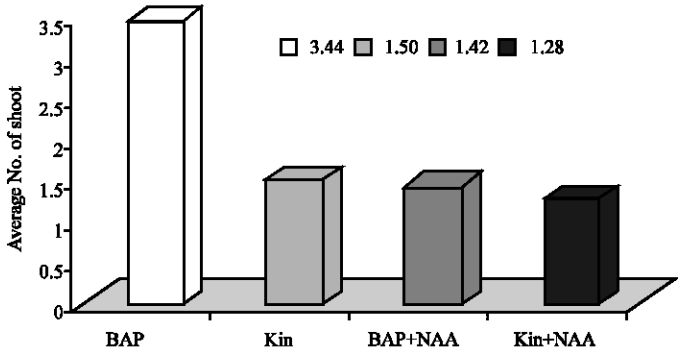

Fig. 3: Average number of multiple shoot formation under different plant growth regulators (overall concentration and combination)

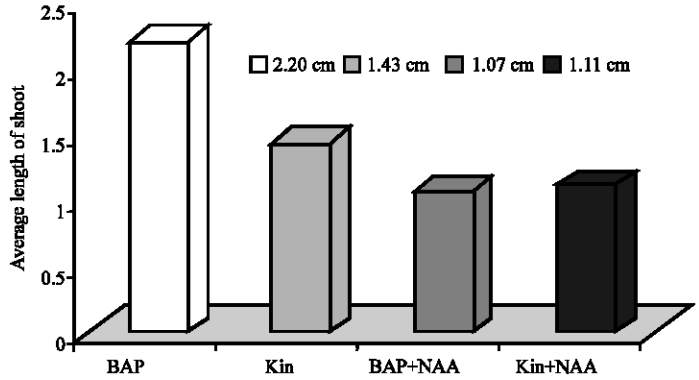

Fig. 4: Average length of shoot (in cm) under different plant growth regulators (overall concentration and combination)

Table 2: Effect of different concentrations of NAA and 2,4-D on callus induction from leaf explants. Each treatment consisted of 10 explants and data were recorded after 6 weeks of culture

Growth regulators (mg L^{-1})	Percentage of explants induced callus	Degree of callus formation	Texture and color of callus
NAA			
0.1	60.00	+	Cr.
0.2	70.00	+	Cr.
0.5	80.00	++	Cr.
1.0	90.00	+++	S, Cr.
2.0	80.00	++	S, Cr.
5.0	60.00	+	Cr.
2,4-D			
0.1	-	-	-
5.0	-	-	-
1.0	40.00	+	Br. H
2.0	50.00	+	Br. H
3.0	30.00	+	Br. H
5.0	-	-	-

+ = Poor, ++ = Moderate, +++ = Massive, - = No callus formation, Cr. = Creamy, S = Soft, Br. = Brown, H. = Hard

Among the all concentrations of NAA, 90% of the cultures induced callus formation in 1.0 mg L^{-1} NAA. The calli were cream in color, soft in nature and degree of callus formation was massive (Fig. 1B). The results are in agreement with the observation of Rahman *et al*. (1992), De Bruijne *et al*. (1974), Yie and Liaw (1977), Arora and Sing (1978a, b) for *Carica papaya*. In the present study 2,4-D was not effective as NAA. The calli was brown in color, hard in texture and degree of callus formation was poor.

Callus Regeneration

Regeneration of shoots occurred when calli were transferred on MS medium with different concentrations (0.1, 0.2, 0.5, 1.0, 2.0, 4.0 and 5.0 mg L^{-1}) of BAP. Eighty percent of shoots regeneration was obtained within 15-20 days when the calli cultured on 0.2 mg L^{-1} BAP and the average number of shoots per callus was 4.25 in formulation (Table 3, Fig. 1C). The results in the present study are corroborated with the observation of Banu *et al*. (1999) in nodal segment callus for *O. sanctum*, Ahmad *et al*. (1993) for internode callus of *Melia azadarch*, Begum *et al*. (1999) and Sitakanta and Chand (1996) for shoot proliferation of *Ocimum sanctum*.

Rooting Formation

The shoots were transferred to rooting medium containing MS salts with different concentrations (0.1, 0.2, 0.5, 1.0 and 2.0 mg L^{-1}) of NAA, IAA and IBA. In the present study, NAA was found more effective for root induction than IAA and IBA (Table 4). In the medium containing higher concentrations of NAA, IAA and IBA also produced callus around the root zone. The results are in agreement with the observation of Sooch *et al*. (1998) for *Dianthus caryophyllus* L. Roberts *et al*. (1990) reported that roots, which originated from callus known to develop poor vascular connections with the stem. However 90% of shoots produced root in 0.1 and 0.2 mg L^{-1} NAA. Maximum average number of root per culture was 5.66 and average length of root was 3.55 cm in 0.1 mg L^{-1} NAA after eight weeks of culture (Fig. 1D).

The results are in agreed with the observation of *Ocimum basilicum* L. (Begum *et al*., 2002) *Ruscus hypophyllum* (Jha and Sen, 1985), *Dendrobium* CV (Singh *et al*., 1998; Honmode, 1992; Sharon and Vasundhara, 1990) and *Rouwolfia* micrantha (Sudha and Seeni, 1996). Among the all concentrations of IAA 50% of shoots produced root in 0.1 mg L^{-1} IAA. Maximum average number of root per culture was 3.20 and average length of root was 3.10 cm in 0.1 mg L^{-1} IAA. On the other hand among the all concentrations of IBA 0.1 mg L^{-1} IBA was effective for root induction than other

Table 3: Effect of different concentrations of BAP on plant regeneration from leaf derived callus. Each treatment consisted of 10 explants and data were recorded after 8 weeks of culture

Growth regulators (mg L^{-1})	Percentage of shoot formation	Average No. of shoot per callus	Average length of shoot per callus (in cm)
BAP			
0.1	50.00	3.20	1.20
0.2	80.00	4.25	1.37
0.5	40.00	2.00	0.87
1.0	20.00	1.50	0.75
2.0	-	-	-
4.0	-	-	-
5.0	-	-	-

Table 4: Effect of different concentrations of auxins on root induction from regenerated shoots. Scoring was done after 8 weeks of culture and each treatment consists of 10 explants

Growth regulators (mg L^{-1})	Percentage of root induction	Average No. of root per culture	Callus induction	Average length of root (in cm)
NAA				
0.1	90.00	5.66	-	3.55
0.2	90.00	5.22	-	3.11
0.5	70.00	3.42	+	2.85
1.0	30.00	2.33	+	1.83
2.0	-	-	++	-
IAA				
0.1	50.00	3.20	-	3.10
0.2	40.00	2.25	-	2.75
0.5	30.00	2.00	-	2.66
1.0	10.00	1.00	+	1.50
2.0	-	-	+	-
IBA				
0.1	80.00	3.75	-	4.06
0.2	60.00	3.50	-	3.33
0.5	50.00	3.20	-	3.00
1.0	-	-	+	-
2.0	-	-	++	-

+ = Poor, ++ = Moderate callus formation

concentration use. The highest 80% of shoots produced root in 0.1 mg L^{-1} IBA. Maximum number of root per culture was 3.75 and maximum length of root was 4.06 cm in 0.1 mg L^{-1} IBA. The *in vitro* plantlets were successfully established on the soil and 75% of them were survived (Fig. 1E).

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