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IN VITRO REGENERATION OF POPULAR TOBACCO VARIETIES OF BANGLADESH FROM LEAF DISC

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Abstract

Regeneration ability of five *Nicotiana* varieties viz., Virginia, Jati, Motihari, CC Bengal and Sumatra were investigated via callus induction using leaf discs. Explants were cultured on MS medium supplemented with different concentrations and combinations of plant growth regulators. Callus formation frequency was 67.20%. Among the varieties used, Motihari induced the highest percentage (97.50%) of callus followed by Jati (92.50%) in 2.0 rng/L Kinetin and 2.0 mg/L IAA. Shoots were induced from calli cultured on the same medium. Maximum shoot formation from leaf discs was 82.50% on medium supplemented with 2.0 mg/L Kinetin and 2.0 mg/L IAA. It was also revealed from this study that Motihari was the best variety for callus formation and subsequent plantlet regeneration which is a pre-requisite for vector mediated transformation for varietal improvement of *Nicotiana* species. The rooting response of regenerated shoots was observed by using $^{1}/_{2}$ MS medium with IBA (0.0, 0.5, and 1.0 mg/L). The highest root formation was found in Motihari (90%) with ½ MS medium supplemented with 0.5 mg/L IBA. After that regenerated plantlets with plenty of roots were transferred successfully to pots and subsequently to the field.

Keywords: Tobacco, *Nicotiana, in vitro* regeneration, callus induction, plantlet regeneration, leaf disc, phytohormone.

Introduction

Tobacco *(Nicotiana* species) is an ancient and the most important and widely grown commercial non-food crop in the world. This is also a major tropical cash crop of considerable economic significance to Bangladesh. The plant tobacco belongs to the genus *Nicotiana* under the large family Solanaceae, the nightshade family (Garner, 1951). There are only two cultivated species under this genus *viz., Nicotiana tabacum* and *Nicotiana rustica* which was established by Carolas Linnaeus in 1753. There are few mentionable tobacco varieties of Bangladesh, such as Virginia, Jati, Motihari, CC Bengal, Sumatra, etc. Motihari belongs to *Nicotiana rustica* and the others belong to *Nicotiana tabacum.* Virginia and Sumatra are two recognized cigarette varieties, while others are used for Hokka, Bidi, and Zarda purpose.

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In Bangladesh, tobacco has a prestigious and significant position in terms of economy where about 2-3 million people are employed for its production, processing and marketing. Tobacco earns foreign currency which occupies 4th position after jute, sugarcane, and tea. The land under tobacco cultivation is 76110 acres and production is 36840 tons (BBS, 2004).

Tobacco has been used as a model crop plant for *in vitro* studies on regeneration, since the classical studies of Skoog and Miller (1957). Totipotency was first demonstrated with *Nicotiana tabacum* by regeneration of mature plants from single cells (Vasil and Hildebrandt, 1965). Variability of regenerates has been obtained from tobacco tissue culture (Nikova and Zagorzka, 1984). Advanced quality hybrids and asymmetrical hybrids through protoplast fusion (Kortash and Kanevsku, 1987), resistance to herbicide (Freyssinet, 1986), obtaining TMV mosaic free plantlets through tobacco callus culture (Sanger *et al.,* 1986), overcoming cross incompatibility and obtaining male sterile forms (Nikova *et al.,* 1988), demonstration of gene transformation (Zhang *et al.,* 1998) and determining effect of antibodies on the *in vitro* growth response (Silva *et al.,* 2003) have been successfully obtained from tobacco tissue culture.

So, there is no doubt that *in vitro* regeneration in tobacco has the great potentiality for its improvement. Although tissue culture techniques in tobacco have been successful long ago, but in Bangladesh, its application is limited. Considering the above mentioned information, the present study was undertaken to standardize the different hormonal concentrations for tobacco callusogenesis and plantlet regeneration using leaf disc to observe the callus induction potentiality in five tobacco cultivars.

Methodology

Experimental materials & media

The seed materials of five tobacco cultivars *viz.,* Virginia, Jati, Motihari, CC Bengal, and Sumatra were used in this study. Different culture media were used in the present investigation for various purposes. MS (Murashige and Skoog, 1962) medium containing 1.0, 1.5, 2.0, 2.5, and 3.0 mg/L kinetin in association with 2.0 mg/L was used for callus induction and subsequent plantlet regeneration. Hormone free $^{1}/_{2}$ MS medium and $^{1}/_{2}$ MS medium with IBA (0.5 and 1.0 mg/L) were used to observe the rooting responses of regenerated shoots.

Sterilization of experimental materials

Various chemicals were used for tobacco seed sterilization. Out of them, the most frequently used ones are 70% ethyl alcohol, Clorox 40%, and 0.1% $HgCl_2$. At the very first stage, seeds were washed by running tap water for 5-10 minutes. After washing, seeds were kept in sterile vials or beaker for several minutes to separate

the floating seeds from the fresh ones. The floating seeds were strongly discarded. After that the seeds were rinsed in 70% ethyl alcohol for eight minutes and washed by doubled distilled water. During this course, vials were shaken vigorously. Thereafter, the seeds were forwarded to wash in 0.1% $HgCl_2$ and Clorox (40%). Clorox (40%) treated seeds gave the better results than 0.1% $HgCl_2$. Water was then drained out from the seeds. Then the seeds were washed by dd H_2O for six times and kept in water for around 30 minutes which facilitated better germination.

Individual vials or petridishes were used for individual varieties. Except washing the seeds in running tap water, all the sterilizing activities were done in the laminar airflow cabinet with care.

Culture Methods

1) Axenic culture

Sterilized seeds were placed on seed germination medium in vials. In each vial 10-15 seeds were placed, the culture was then incubated in incubation room till the germination of seeds. The age of the seedling used for explants were 10 -15 days.

ii) Explants culture

The seedlings raised in axenic culture were used as the source of explants. Leaf discs around 2 mm were used as explants. The aseptically grown 12 days old seedlings were rescued and placed on a sterile glass plate. Comparatively large and mature leaves were separated from the seedlings. The separated leaves were then cut into many small segments with the help of sterile scalpel and forceps and placed upright with cut ends embedded in the sterile culture medium for callus induction. It was also mentioned that different concentrations and combinations of growth regulators like Kinetin and IAA were used for callus induction and regeneration from callus. In each petridish, 4-5 leaf segments were placed.

iii) Subculture

Two weeks after inoculation of explants, the calli attained convenient size, they were removed aseptically from the vials on a sterilized glass plate inside the laminar air flow cabinet and were placed again on freshly prepared medium containing appropriate hormonal supplements for shoot induction from the cells. The sub culturing media in the present investigation were MS containing different combinations of Kinetin and IAA. The sub cultured vials were again incubated at 22 ± 2°C with 16 hours photoperiod. After shoot initiation, more light intensity was used for shoot elongation. The culture vessels showing the sign of contamination were discarded. Repeated subcultures were done at an

interval of 15 days and incubated under the same temperature as mentioned previously for maintenance of calli and organogenesis.

iv) Rooting

The subcultured calli continued to proliferate and differentiate into shoots grew about 3-4 cm in length, they were rescued aseptically from the cultured vials and were separated from each other and again cultured individually on vials with freshly prepared root induction medium supplemented with different concentrations of IBA to induce root. The vials containing plantlets were incubated at 22 ± 2°C with 16 hrs photoperiod. Day to day observations were carried out to note the responses.

Preparation of pot and transplantation

Potting mixture containing garden soil, sand, and cowdung in the ratio 1:2:1 was mixed properly and autoclaved one hour in 121°C for 30 minutes at 1.16 kg/cm^2. After cooling, the soil mixture was taken into 10 cm plastic pots for growing the plantlets at *in vivo* condition.

When the plantlets became *5-8* cm in height with sufficient root system, they were taken out from the vials. Medium attached to the roots was gently washed out running tap water. The plantlets were then transplanted to pot containing potting mixture mentioned above. Immediately after transplantation, the plants along with the pots were covered with moist polythene bag to prevent desiccation. To reduce sudden shock, the pots were kept in a growth room for 7-15 days under controlled environments. The interior of the polythene bags was sprayed with distilled water at every 24 hrs to maintain high humidity around the plantlets. At the same time, plantlets were also nourished with Hogland's solution. After two to three days, the polythene bags were gradually perforated to expose the plants to natural environment. The polythene bags were completely removed after ten to fifteen days when the plantlets appeared to be self-sustainable. At this stage, the plantlets were placed in natural environment for 3-10 hours daily. Finally, after 15-20 days, they were transferred to the field.

Results and Discussion

Leaf discs of 12-day old seedlings of five *Nicotiana* cultiovars were cultured on MS medium supplemented with five different concentrations of Kinetin along with constant concentration of IAA to induce callus. Callus initiation started from 6.00 to 10.60 days of incubation in Motihari and Sumatra, respectively.

It was found that out of the five varieties, Motihari showed the highest callus induction (100.00 %) which required minimum (6.0) days for callus initiation (Showed in plate 1), followed by Jati (95.00%), Virginia (90.00%), CC Bengal

(80.00%), and Sumatra showed the lowest callusing (70.00 %) on MS + 2.0 mg/L Kinetin + 2.0 mg/L IAA (T_3) and the lowest callusing was observed in T_1 (MS + 1.0 mg/L Kinetin + 2.0 mg/L IAA). Callus induction performances of all the varieties in each treatment were evaluated and presented in the Table 1. A wide range of variation in callus induction ability i.e., in different callus characters were observed on different treatments (Table 2). The highest callusing per petridish (3.90) was found in Motihari on MS medium + 2.0 mg/L Kinetin + 2.0 mg/L IAA followed by Jati (3.70) and the lowest callusing was found in Sumatra (1.30) on MS + 1.0 mg/L Kinetin + 2.0 mg/L IAA. In case of callus size, T_3 with Motihari showed biggest size of callus (1.198cm) and smallest size callus was found in Sumatra (0.447cm) with T_1. Most of the hormone x genotype interactions gave almost moderate abundance of callus.

Table 1. Effects of different concentrations of Kinetin in combination with MS + 2.0 mg/L IAA on callus induction from leaf discs of five *Nicotiana* varieties.

Hormonal concentration	Variety	No. of explants incubated	% Calls induction	Days required for callus initiation
1.0 mg/L Kinetin (T_1)	Virginia	20	50	9.400
	Motihari	20	60	7.400
	Jati	20	55	8.400
	CC Bengal	20	40	9.600
	Sumatra	20	35	10.60
1.5 mg/L Kinetin (T_2)	Virginia	20	80	8.400
	Motihari	20	90	6.400
	Jati	20	85	7.400
	CC Bengal	20	70	8.400
	Sumatra	20	60	9.400
2.0 mg/L Kinetin (T_3)	Virginia	20	90	8.200
	Motihari	20	100	6.000
	Jati	20	95	7.200
	CC Bengal	20	80	8.200
	Sumatra	20	70	9.200
2.5 mg/L Kinetin (T_4)	Virginia	20	70	8.600
	Motihari	20	80	6.400
	Jati	20	75	7.200
	CC Bengal	20	60	8.600
	Sumatra	20	50	9.600
3.0 mg/L Kinetin (T_5)	Virginia	20	60	9.600
	Motihari	20	70	7.200
	Jati	20	65	8.400
	CC Bengal	20	50	9.400
	Sumatra	20	40	10.40

Table 2. Performance of the hormone × variety interaction on different callus characteristics of tobacco.

Hormone × variety		Callusing per petridish	Sizze of callus (cm)	Days required for callus initiation
1.0 mg/L Kinetin (T_1)	Virginia	1.90 g-i	*0.6520* 1	Poor
	Motihari	2.30 e-h	0.7970 ij	Moderate
	Jati	2.10 f-h	0.7650jk	Moderate
	CC Bengal	1.50 hi	0.5710m	Poor
	Sumatra	1.30 i	0.4470 n	Poor
1.5 mg/L Kinetin (T_2)	Virginia	3.10 b-e	0.9210 e	Moderate
	Motihari	3.50 a-c	1.096 b	Plenty
	Jati	3.30 a-d	1.002 c	Plenty
	CC Bengal	2.70 d-g	0.8300 hi	Moderate
	Sumatra	2.30 e-h	0.7380 k	Moderate
2.0 mg/L Kinetin (T_3)	Virginia	3.50 a-c	1.0l0 c	Plenty
	Motihari	3.90 a	1.198 a	Plenty
	Jati	3.70 ab	1.110 b	Plenty
	CC Bengal	3.10 b-e	0.9600 d	Moderate
	Sumatra	2.70 d-g	0.8540 gh	Moderate
2.5 mg/L Kinetin (T_4)	Virginia	2.70 d-g	0.8220 hi	Moderate
	Motihari	3.10 b-e	0.9870 cd	Plenty
	Jati	2.90 c-f	0.9110 ef	Moderate
	CC Bengal	2.30 e-h	0.7300 k	Moderate
	Sumatra	1.90 g-i	0.6780 1	Moderate
3.0 mg/L Kinetin (T_5)	Virginia	2.30 e-h	0.7290 k	Moderate
	Motihari	2.70 d-g	0.8760 fg	Moderate
	Jati	2.50 d-g	0.8 150 hi	Moderate
	CC Bengal	1.90 g-i	0.6500 1	Poor
	Sumatra	1.50 hi	0.5620 m	Poor

While the callus formation was completed, different combination and concentrations of Kinetin and constant concentration of IAA in MS medium were used to observe the shoot regeneration capacity of the calli of different varieties. Shoot regeneration of five *Nicotiana* varieties showed a distinct range of variation against each and every concentration and combination of phytohormones (Table 3). Percentage of shoot regeneration and days to shoot regeneration were different in different treatments and genotypes. The highest shoot regeneration was found in Motihari (95.00%) under T_3 (MS + 2.0 mg/L Kinetin + 2.0 mg/L IAA) and the lowest was found in Sumatra (40%) with T_1 (MS + 1.0 mg/L Kinetin + 2.0 mg/L IAA).

Table 3. Response of different combinations and concentrations of phytohormones on shoot regeneration from leaf discs of *Nicotiana* varieties.

Supplements	Varieties	Number of callus inoculated	% Shoot regeneration	Days to shoot regeneration
MS +1.0 mg/L Kinetin (T_1)	Virginia	20	60.00	21.60
	Motihari	20	75.00	19.60
	Jati	20	70.00	20.60
	CC Bengal	20	50.00	22.60
	Sumatra	20	40.00	23.60
MS +1.5 mg/LKinetin (T_2)	Virginia	20	75.00	20.40
	Motihari	20	90.00	18.40
	Jati	20	*85.00*	19.40
	CC Bengal	20	70.00	21.40
	Sumatra	20	65.00	22.40
MS + 2.0 mg/L Kinetin (T_3)	Virginia	20	80.00	20.20
	Motihari	20	95.00	18.20
	Jati	20	80.00	19.20
	CC Bengal	20	75.00	21.20
	Sumatra	20	70.00	22.20
MS + 2.5 mg/L Kinetin (T_4)	Virginia	20	70.00	20.60
	Motihari	20	85.00	18.60
	Jati	20	80.00	19.60
	CC Bengal	20	60.00	21.60
	Sumatra	20	55.00	22.60
MS +3.0 mg/L Kinetin (T_5)	Virginia	20	65.00	21.40
	Motihari	20	80.00	19.40
	Jati	20	75.00	20.40
	CC Bengal	20	55.00	22.40
	Sumatra	20	50.00	23.40

MS medium with different concentrations of IBA (0.0, 0.5, and 1.0 mg/L) were used to observe the rooting responses of regenerated shoots. Root initiation ability also varied in a wide range due to difference of IBA concentrations as well as differences in genotypes. The highest root initiation was observed in leaf discs of Motihari (90.00%) with T_2 ($^1/_2$ MS medium supplemented with 0.5 mg/L IBA) and the lowest was found in Sumatra (10.00%) with T_1 (Hormone free $^1/_2$ MS medium) are shown in the Table 4.

Table 4. Effect of hormone free MS medium and different concentrations of IBA in half strength of MS medium for root initiation *of Nicotiana* varieties.

Supplements	Varieties	Number of shoots inoculated	Number of Shoots with roots	% Root formation	Days to root initiation
$^1/_2$ MS medium without hormone (T_1)	Virginia	10	3.0	30.00d-f	12.20c
	Motihari	10	5.0	50.00 b-f	10.20 ef
	Jati	10	4.0	40.00 c-f	11.20d
	CC Bengal	10	2.0	20.00 ef	13.20 b
	Sumatra	10	1.0	10.00f	14.20a
$^1/_2$ MS + 0.5 mg/L 1BA (T_2)	Virginia	10	7.0	70.00a-d	9.200 g
	Motihari	10	9.0	90.00ab	7.200 k
	Jati	10	8.0	80.00a-c	8.000 ij
	CC Bengal	10	6.0	60.00a-e	10.20 ef
	Sumatra	10	5.0	50.00b-f	11.20d
$^1/_2$ MS + 1.0 mg/L IBA (T_3)	Virginia	10	6.0	60.00a-e	9.400fg
	Motihari	10	8.0	80.00a-c	8.200 i
	Jati	10	7.0	70.00a-d	8.400 hi
	CC Bengal	10	*5.0*	50.00b-f	10.40e
	Sumatra	10	4.0	40.00c-f	11.60 cd

After satisfactory development in root system, the small plantlets were removed from the culture media and transplanted in small plastic pots containing sterile soil, sand, and cowdung in a 1:2:1 ratio in growth chamber for proper hardening of the plantlets (Plate-5) and finally those were planted in earthen pots (Plate 6). Gradually, the plantlets were adapted to the soil. The survival rate of plantlets of Virginia, Motihari, Jati, CC Bengal, and Sumatra were 75.00, 77.77, 71.42, 62.50 and 57.14% in the pots and 83.33, 85.00, 80.00, 60.00, and 50.00% in the soil, respectively. The survived plants then showed vigorous growth with proper leaf development. Comparative survivability of regenerants obtained from leaf discs of five genotypes of *Nicotiana* after transferring them in pot and in soil are shown in the Table 5.

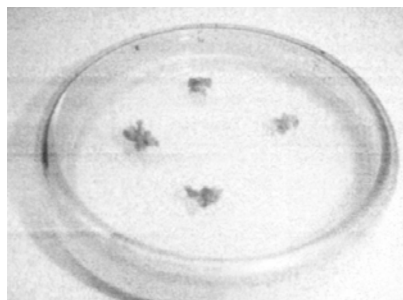

Plate 1. Callus induced form leaf explants of the variety Motihari on MS medium containing 2.0 mg/L kinetin + 2.0 mg/L IAA

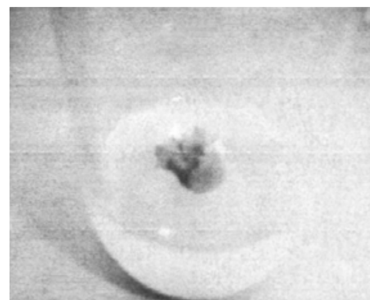

Plate 2. Shoot initiation from leaf discs of Motihari on 2.0 mg/L kinetin + 2.0 mg/L IAA

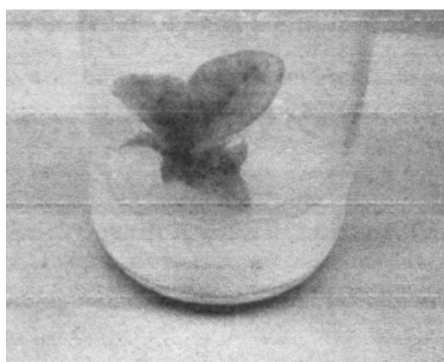

Plate 3. Regenerated shoot of Virginia on 2.0 mg/L Kinetin + 2.0 mg/L IAA

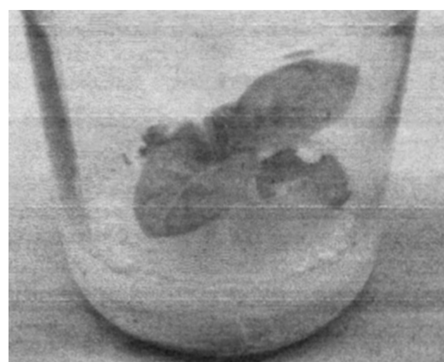

Plate 4. Initiation of rrots from regenerated shoots of CC Bengal in ½ MS medium ontaining 0.5 mg/L IBA

Plate 5. Acclimatized plantlet of Motihari in plastic pot

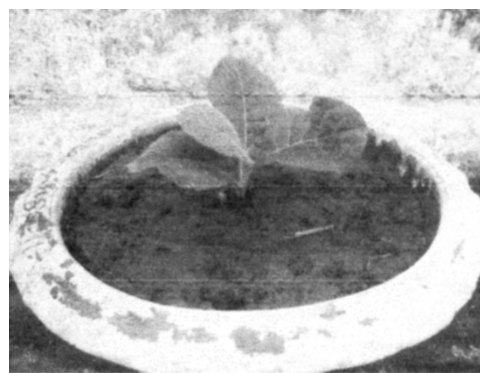

Plate 6. Regenerated plantlet of the variety Jati in earthen pot

A wide range of variation in *in vitro* regeneration potentiality of the genotypes was observed on different treatment combinations on MS media. Induced calli ranged from 35-100%. Average callusing was the highest in Motihari (77.50%) followed by Jati (72.50%), Virginia (67.50%), CC Bengal (57.50%), and Sumatra (48.50%). Callusing was also highest (84.50%) in MS + 2.0 mg/L Kinetin + 2.0 mg/L IAA (T_3) and the lowest in MS + 1.0 mg/L Kinetin + 2.0 mg/L IAA (T_1). Responses of shoot formation of different genotypes were different. Among the investigated genotypes, the regeneration performances were found better in Motihari (87.50%) followed by Jati (77.50%) in T_3 (MS + 2.0 mg/L Kinetin + 2.0 mg/L IAA). Therefore, considering callus induction, shoot regeneration and rooting performances, Motihari was found more potential for *in vitro* regeneration than the other four varieties.

Table 5. Survival rate of regenerants obtained from explants of five varieties of *Nicotiana* species in pot and soil.

Planting condition	Name of variety	Number of plants transplanted	Number of plants survived	Survival rate (%)
In Pot	Virginia	8	6	75.00
	Motihari	9	7	77.77
	Jati	7	5	71.42
	CC Bengal	8	5	*62.50*
	Sumatra	7	4	57.14
In Soil	Virginia	6	5	83.33
	Motihari	7	6	*85.00*
	Jati	5	4	80.00
	CC Bengal	5	3	60.00
	Sumatra	4	2	50.00

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