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Efficient Regeneration of Tobacco (*Nicotiana tabacum* L.) Plantlets from Cotyledon, Hypocotyl and Leaf Explants: An Excellent Model Plant for Gene Function Analysis

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Authors' contributions

This work was carried out in collaboration among all authors. Author MS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors KF and MARS managed the analyses of the study. Author AR managed the literature searches. Author SMR supervised the whole process. All authors read and approved the final manuscript.

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ABSTRACT

Tobacco has been widely used as a model plant for stable and non-stable gene function analysis. Successful *Agrobacterium*-mediated transformation mainly depends on *in vitro* regeneration of tobacco plant. However, a reliable and standard regeneration protocol of tobacco using multiple explants is limited. In this study, we established a reliable and reproducible regeneration protocol of tobacco using three different explants i.e. cotyledon, hypocotyl and leaf. Preliminary, surface sterilized tobacco seeds were germinated on growth regulator free MS medium. Thereafter, *in vitro* germinated explants were inoculated into Murashige and Skoog [1] media supplemented with different combination and types of growth regulators for callus induction and subsequent regeneration of plantlets. It was revealed that, regeneration ability of explants is greatly influenced by type and nature of the explant. Among the three explants, higher callus induction (95%) was

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obtained in MS medium supplemented with 2.0 mg l^{-1} kinetin + 2.0 mg l^{-1} IAA from leaf explant. Also, leaf explant exhibited much higher regeneration ability (95%) than hypocotyl (60%) and cotyledon (45%) explants. Significantly highest number of shoots (8.0) were regenerated from leaf explants cultured on MS medium supplemented with 3.0 mg l^{-1} Kinetin+1.0 mg l^{-1} IAA compared to the other hormone combinations. Regenerated mature shoots were showed normal root after transferred onto ½ MS medium containing 0.3 mg l^{-1} IBA. This study will provide valuable information related to *in vitro* regeneration of tobacco plantlets using cotyledon, hypocotyl and leaf explants and will be used as a standard protocol for *Agrobacterium*-mediated transformation for gene function analysis.

Keywords: MS medium; leaf; callus induction; in vitro regeneration; acclimatization.

1. INTRODUCTION

The tobacco (*Nicotiana* sp.) belongs to the family Solanaceae. There are about 69 species of *Nicotiana* genus which are available in the world [2,3]. Mainly two tobacco species namely *N. tabacum* and *N. rustica* are cultivated throughout the world for commercial purpose. Tobacco contains about 2500 types of major alkaloids and terpenoids, all of which are valuable for their variety of important medicinal properties [4]. Tobacco is also cultivated commercially in Bangladesh. Commercial varieties of tobacco in Bangladesh are Virginia, Jati, Motihari, CC Bengal, and Sumatra [5].

N. tabacum has been extensively used by many researchers as a model plant for studying morphogenesis, physiology, metabolism, and genetics [6,7,8]. Cellular totipotency of a plant cell was first demonstrated with *N. tabacum* by *in vitro* regeneration of mature plants from single cells [6]. At present, this plant species due to its high transformation efficiency, is being used as a model organism for molecular firming and for the overexpression of a wide variety of proteins [9,10,11]. Also *in vitro*-derived tobacco calli is necessary to study the biosynthesis of different types of bio-pharmaceuticals. The callus culture of tobacco has been proved extremely useful for studying the cellular basis of morphogenesis and has enabled the complex influence of hormones on this process. Transgenic tobacco plant production is also necessary for the research of various types of agronomically important crop characters. Therefore, callus induction and regeneration from various suitable explants of tobacco has facilitated the improvement of this crop.

In this study, we attempted to standardize suitable culture media formulation for callus induction, plant regeneration, shoot multiplication, and successful acclimatization of *N. tabacum* var. Virginia to establish an efficient and reproducible *in vitro* mircro-propagtion protocol which would be useful in morphogenesis, genetic transformation and bio-firming study in this plant species.

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

We used three explants such as cotyledon, hypocotyl and leaf of *N. tabacum* var. Virginia as experimental plant materials in this research work. We purchased the seeds of *N. tabacum* var. Virginia from the commercial market of Khustia Sadar, Bangladesh. This research work was conducted in the central laboratory, Department of Genetic Engineering and Biotechnology, Jashore University of Science and Technology, Jashore 7408, Bangladesh in 2019.

2.2 Seed Sterilization and *in vitro* Germination

Disease free, healthy and viable *N. tabacum* var. Virginia seeds were surface-sterilized by rinsing in 70% ethanol for 30 sec and then washed more than three times in sterile distilled water. Seeds were further washed by 2-3 drops Savlon (ACI Company Ltd., Bangladesh) with sterile distilled water for five minutes with vigorous shaking followed by sterile distilled water till the foam was completely removed. Then, seeds were taken into sterile 1.5 ml eppendrop tube and suspended in 0.1% $HgCl_2$ solution for different treatment period to ensure contaminant free culture. Immediately, surface sterilized seeds were thoroughly washed 6-7 times with sterile distilled water. Then the seeds were placed in a culture tube containing growth regulator free MS medium for seed germination. The seeds were incubated at culture room conditions (temperature 25±2°C, light condition 16 h/8 h, intensity 2000 lux and relative humidity 60-65%).

2.3 Preparation of Explants

For all the experiments 3 weeks old *in vitro* germinated seedlings were used as source of explants i.e. cotyledon, hypocotyl and leaf. About 1 × 1 cm size explants were used as initial culture and placed up abaxial surface touching the culture medium (Fig. 1).

2.4 Culture Media and Explants Growth Condition

Murashige and Skoog [1] medium with standard amounts of vitamins, 3% (w/v) sucrose, 0.5% (w/v) phytoagar was used in all the culture media. The medium pH was adjusted to 5.70 by 1 M NaOH or 1 M HCl. Then, the medium was autoclaved at 121°C for 20 min at 15 psi of pressure. As synthetic plant hormone like BAP, NAA are heat stable, those hormones were added before autoclaving of the media but natural hormone like kinetin are heat labile and was added to the media through filter sterilization in laminar airflow cabinet after autoclaving. All culture medium was maintained at previously described culture room condition.

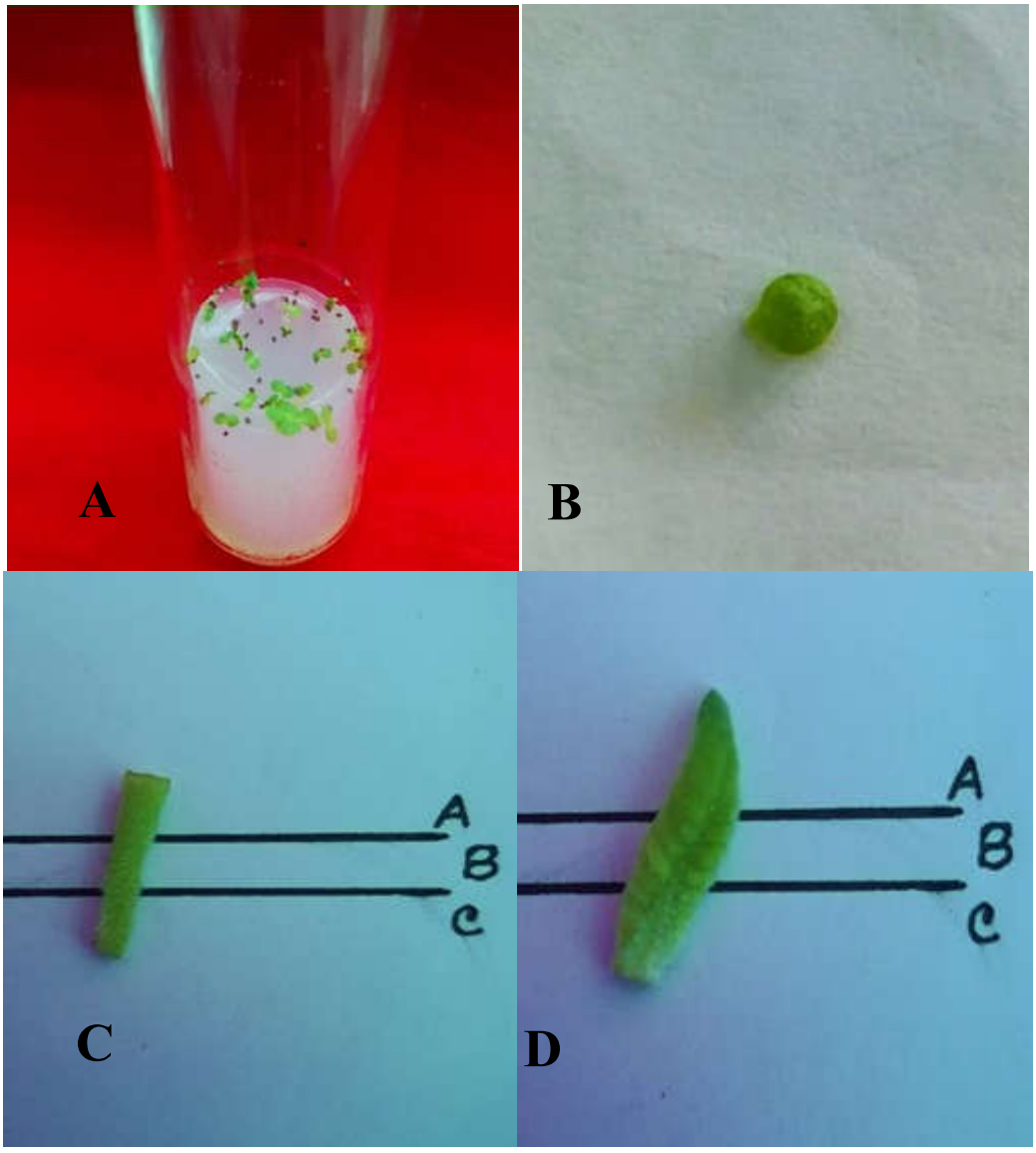

Fig. 1. Preparation of explants for *in vitro* regeneration. (A) *N. tabacum* seeds germination on hormone free MS medium. (B) Cotyledon (C) Hypocotyl and (D) Leaf explants were used in this study

3. RESULTS AND DISCUSSION

3.1 Surface Sterilization of Tobacco Seeds for *In vitro* Culture

To ensure aseptic condition for *in vitro* seed germination, it is very essential to conduct surface sterilization of seeds before inoculation. Surface sterilization of collected seeds was carried out by standard 0.1% $HgCl_2$ solution at different time duration ranges from 1.0- 4.0 minutes. Data on seeds survival rate of virginia variety is presented in Table 1. When the seeds were treated with 0.1% $HgCl_2$ solution for 1.0 minute, higher fungal contamination (80%) occurred as the treatments failed to kill the microorganisms presented on the surface of seeds. When 0.1% $HgCl_2$ solution was used for 4 minute, 85% seeds were damaged due to long exposure of $HgCl_2$. When 0.1% $HgCl_2$ solution was used in short duration, they failed to kill the microorganisms attached to the surface of the seeds. As a result, maximum number of cultures was contaminated within 2 weeks of inoculation. When 0.1% $HgCl_2$ solution was used in long duration, it showed some efficiency but severely occurred tissue killing. The maximum survival rate (95%) of seeds was observed for 3 minutes treatment duration.

In one- way ANOVA, If F > F crit, the means of several populations are not all equal. Here, it is observed that there is statistically significant difference in seed germination due to variation of treatment duration determined by one-way ANOVA ($F = 12.718 > F\ crit = 4.747$; $p = 0.004$) at significance level (denoted as α or alpha) of 0.05.

To develop contamination free seeds, removal of the seed surface contaminants using different sterilants is very important. For surface sterilization of seeds, many researchers used many types of sterilizing agents with different concentrations. The treatment may include 1% solution of sodium hypo chloride, 70% alcohol, 0.1% $HgCl_2$ solution, 1% silver nitrate solution. There are many reports of using $HgCl_2$ [12,13,14] for surface sterilization of the seeds. Druart and Gruselle [15] described that concentrations of disinfectants and suspended times are adjusted according the sensitivity of explants to sterilants. In this experiment, 0.1% $HgCl_2$ solution was used for different duration of time for seeds. Long exposure of the seeds with $HgCl_2$ solution greatly hampered the germination ability of the seeds. Altaf et al. [16] also reported the harsh effects of the sterilants resulting very slow growth of cultures from sterilized seeds.

3.2 Callus Induction from Cotyledon, Hypocotyl and Leaf Segment of Tobacco

The explants of younger age are more regenerative than the older aged explants as the juvenile cells are having more totipotency. For callus induction, the position of explant is also an important factor. Middle portion of leaf segments showed maximum results during callus formation because middle portions are easily attached with media that facilitate to uptake of nutrients from media.

The culture of MS medium supplemented with BAP with IAA or NAA, and kinetin with IAA was employed for callus induction from cotyledon, hypocotyl and leaf segment explants in the light condition. Almost all the concentrations and combinations were observed in inducing callus but the callogenic response of the explants were found to vary with different doses of hormonal combinations. The results obtained are shown in Table 2 and Fig. 2(A, B, C).

Table 1. Effects of 0.1% $HgCl_2$ for different duration on surface sterilization *of N. tabacum*

Treatment (Min)	No. of inoculated seeds	No. of contaminated seeds	% of contaminated seeds	No. of germinated seeds	% of germinated seeds
1.0	20	16	80	4	20
1.5	20	9	45	11	55
2.0	20	7	35	13	65
2.5	20	3	15	17	85
3.0	20	1	5	19	95
3.5	20	18	90	2	10
4.0	20	17	85	3	15

In case of cotyledon explants inoculated in the MS media supplemented with 2.0 mg l^{-1} kinetin and 2.0 mg l^{-1} IAA, maximum percentage (45%) of response was recorded for callus formation but when cultured on MS medium with 1.0 mg l^{-1} BAP+0.1 mg l^{-1} NAA, the lowest (5%) of explants responded for callus induction. But when hypocotyl used as explant then best result (65%) was recorded from the combination of 2.0 mg l^{-1} kinetin and 2.0 mg l^{-1} IAA, and the lowest result (35%) was recorded from the combination of 1.0 mg l^{-1} BAP and 0.1 mg l^{-1} NAA. In case of leaf segment, explants inoculated in the MS media supplemented with 2.0 mg l^{-1} kinetin and 2.0 mg l^{-1} IAA, maximum percentage (95%) of response was recorded for callus formation but when cultured on MS medium with 1.0 mg l^{-1} BAP+ 0.1 mg l^{-1} IAA the lowest (60%) of explants responded for callus induction in MS medium.

If F > F crit, the means of several populations are not all equal. Here, ANOVA has been carried out for "% of responded explants for callus induction" for cotyledon, hypocotyl and leaf. It is concluded that there is statistically significant difference in callus induction due to variation of explant determined by one-way ANOVA (F = 31.367 > F crit = 3.682; p = 4.376) at significance level (denoted as α or alpha) of 0.05

In our investigation, it was observed that incubation of culture in light condition was found to be an important factor for induction of callus. Nature and texture of induced callus depend on the types of explants. The nature of callus obtained from cotyledon and leaf was greenish friable and callus from hypocotyl was whitish compact in nature. In this experiment, among different explants, leaf segments exhibited the best response toward callus formation in virginia variety of tobacco used here. The highest percentage of callus was induced on (MS+2.0 mg l^{-1} kinetin+2.0 mg l^{-1} IAA) from all three explants of virginia variety. Rahman et al. [5] also found the highest percentage of callus formation in (MS+2.0 mg l^{-1} IAA+2.0 mg l^{-1} Kinetin) combination. In case of their study hypocotyl and leaf disc explants of tobacco were used as a starting material for callus induction. Maximum callogenesis from hypocotyls and leaf discs was obtained on MS medium supplemented with IAA (2.0 mg l^{-1}), Kinetin (2.0 mg l^{-1}) and it was 90% for both explants which is almost same with our present experiment.

3.3 Shoot Regeneration

While callus formation was completed, different combination and concentration of Kinetin and IAA in MS medium were used to observe the shoot regeneration capacity of the calli. For shoot proliferation, generally the growth regulators like cytokinins influence the process seriously. In consideration of Kinetin, five different concentrations (1.0, 2.0, 3.0, 4.0 and 5.0 mg l^{-1}) and constant concentration (1.0 mg l^{-1}) of IAA were used to test their effects on multiple shoot induction from callus. Results of this study have been tabulated in Table 3 and Fig. 2(D, E, F). In case of cotyledon derived calli, the best response for shoot regeneration was 45% and mean number of shoots per callus was 3 in MS+3.0 mg l^{-1} Kinetin+1.0 mg l^{-1} IAA. For hypocotyl derived calli, the highest percentage (60%) of shoot regeneration was noticed in MS+3.0 mg l^{-1} Kinetin+1.0 mg l^{-1} IAA and the highest number of shoots was 4 at the same media and in case of leaf derived calli, the highest percentage of shoot multiplication (95%) was noticed in MS+3.0 mg l^{-1} Kinetin+1.0 mg l^{-1} IAA. The maximum numbers of shoots per culture was obtained 8 in this concentration within 10-15 days. Lowest percentage of shoot multiplication was 10%, 15% and 50% for cotyledon, hypocotyl and leaf derived calli respectively.

Here, ANOVA has been carried out for "% of responded callus for shoot induction" for cotyledon, hypocotyl and leaf. If F > F crit, the means of several populations are not all equal that means the data are statistically significant. It is concluded that there is statistically significant difference in shoot induction due to variation of explant determined by one-way ANOVA (F = 11.057 > F crit = 3.885; p = 0.002) at significance level (denoted as α or alpha) of 0.05.

From this investigation, it is proved that leaf derived calli is far better than hypocotyl and cotyledon derived calli in case of shoot regeneration. Rahman et al. [5] demonstrated that the highest shoot regeneration was found in MS + 2.0 mg l^{-1} Kinetin + 2.0 mg l^{-1} IAA that justify our experiment although we found the best result in reduced amount of auxin (1.0 mg l^{-1}) IAA.

3.4 Root Induction

The shoot development as well as the rooting of regenerated shoots is especially important for establishing tissue culture derived shoots. Although in most of the cases, regenerated

shoots produce roots spontaneously. Healthy micro-shoots were excised from *in vitro* grown cultures and sub-cultured in MS (hormone free media) and ½ MS medium supplemented with different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 mg l^{-1}) of IBA. *In vitro* regenerated roots in half MS medium was found to be the much longer than those in full MS media. But in full MS medium, most of the culture produced healthy root system with little bit basal callus formation. There is no significant difference among cotyledon, hypocotyl and leaf derived shoots in the stage of their root formation. The highest percentage of root induction was 92% and highest number of root was 6.0 recorded in ½ MS medium supplemented with 0.3 mg l^{-1} IBA in case of shoots derived from leaf explant. For hypocotyl and cotyledon derived shoots the highest percentage root formation was 87% and 85% respectively which are very near to leaf derived shoots. The lowest percentage (40%) for leaf derived shoots and 30% for both hypocotyl and cotyledon was found in the ½ MS with 0.1 mg l^{-1} IBA. Results of this study have been presented in Table 4 and Fig. 2(G, H, I).

Here, ANOVA has been carried out for mean no. of roots for cotyledon, hypocotyl and leaf derived shoots. It is concluded that there is no statistically significant difference in root number due to variation of explant as $F < F$ crit determined by one-way ANOVA ($F = 1.5 < F$ crit $= 3.682$; $p = 0.255$) at significance level (denoted as α or alpha) of 0.05.

Rahman et al. [5] showed that the highest percentage of root initiation was observed with (½ MS medium supplemented with 0.5 mg l^{-1} IBA) that is nearly similar to our findings. But Gowher Ali et al. [17] reported that the roots developed well on hormone free MS media. In that experiment, the highest percentage of root induction was 96% recorded in ½ MS medium supplemented with no hormone.

Table 2. Effects of different concentrations and combinations of plant growth regulators (PGR) for callus induction

Explants	Plant growth regulators (mg l^{-1})	No. of cultured explants	Days of callus initiation	No. of responded explants	% of responded explants	Nature of callus
Cotyledon	BAP+IAA					
	1.0+0.5	20	25-30	3	15	GF
	1.0+0.1	20	28-30	2	10	GF
	Kinetin+IAA					
	2.0+2.0	20	32-40	9	45	GF
	2.5+2.0	20	35-40	4	20	GF
	BAP+NAA					
	1.0+1.0	20	28-30	2	10	GF
	1.0+0.1	20	30-35	1	5	GF
Hypocotyl	BAP+IAA					
	1.0+0.5	20	32-40	12	60	WC
	1.0+0.1	20	30-40	10	50	WC
	Kinetin+IAA					
	2.0+2.0	20	30-35	13	65	WC
	2.5+2.0	20	32-42	12	55	WC
	BAP+NAA					
	1.0+1.0	20	30-45	10	50	WC
	1.0+0.1	20	32-45	7	35	WC
Leaf	BAP+IAA					
	1.0+0.5	20	25-28	13	65	GF
	1.0+0.1	20	28-32	12	60	GF
	Kinetin+IAA					
	2.0+2.0	20	28-35	18	95	GF
	2.5+2.0	20	32-40	10	80	GF
	BAP+NAA					
	1.0+1.0	20	25-29	15	75	GF
	1.0+0.1	20	28-32	14	70	GF

Note: GF= Greenish friable, WC= Whitish compact

Table 3. Effects of different concentration of Kinetin and IAA on multiple shoot regeneration from callus

Explants	Plant growth regulators (mg l-1)	No. of sub culturing callus	Days to shoot initiation	No. of responsive callus	% of responsive callus	Mean no. of shoot per callus (M±SD)
Kinetin+IAA						
Cotyledon	1.0+1.0	20	20-24	2.0	10	1.00±0.00
	2.0+1.0	20	16-19	3.0	15	1.33±0.47
	3.0+1.0	20	13-17	9.0	45	3.00±0.82
	4.0+1.0	20	15-18	6.0	30	2.67±0.47
	5.0+1.0	20	20-24	3.0	15	2.00±0.82
Hypocotyl	1.0+1.0	20	18-20	3.0	15	1.67±0.47
	2.0+1.0	20	14-17	5.0	25	2.33±0.47
	3.0+1.0	20	12-16	12	60	4.00±0.82
	4.0+1.0	20	14-18	9.0	45	3.33±0.94
	5.0+1.0	20	18-22	4.0	20	2.00±0.82
Leaf	1.0+1.0	20	15-20	14	70	2.67±0.47
	2.0+1.0	20	12-18	16	80	4.00±0.82
	3.0+1.0	20	10-15	19	95	8.00±0.82
	4.0+1.0	20	13-17	12	60	5.33±1.25
	5.0+1.0	20	16-20	10	50	3.00±0.82

Table 4. Effects of different concentration of plant growth regulators on root induction

Explants	Plant growth regulators mg l^{-1}	Days to root initiation	% of shoots forming roots	Average no. of roots (M±SD)	Average length of root (cm) (M±SD)
Cotyledon	Full MS_0	15-18	48	2±0.82	1.3±.21
	½ MS+IBA				
	0.1	14-17	30	1±0.82	0.5±0.20
	0.2	13-16	50	3±0.82	1.2±0.29
	0.3	15-17	85	4±1.63	1.9±0.16
	0.4	24-28	78	3±0.82	1.6±0.12
	0.5	29-33	72	2±0.82	0.8±0.16
Hypocotyl	Full MS_0	13-17	50	2±0.82	1.3±0.28
	½ MS+IBA				
	0.1	13-16	30	2±0.82	0.6±0.29
	0.2	12-14	55	3±1.63	1.3±0.22
	0.3	14-18	87	5±1.63	2.0±0.16
	0.4	22-26	80	3±0.82	1.7±0.16
	0.5	28-32	75	2±0.82	0.9±0.08
Leaf	Full MS_0	10-12	55	3±1.41	1.7±0.16
	½ MS+IBA				
	0.1	11-15	40	2±0.82	1.0±0.29
	0.2	10-13	60	4±0.82	1.5±0.16
	0.3	12-15	92	6±1.41	2.5±0.22
	0.4	20-24	85	4±0.82	1.9±0.29
	0.5	26-30	80	3±0.82	1.1±0.14

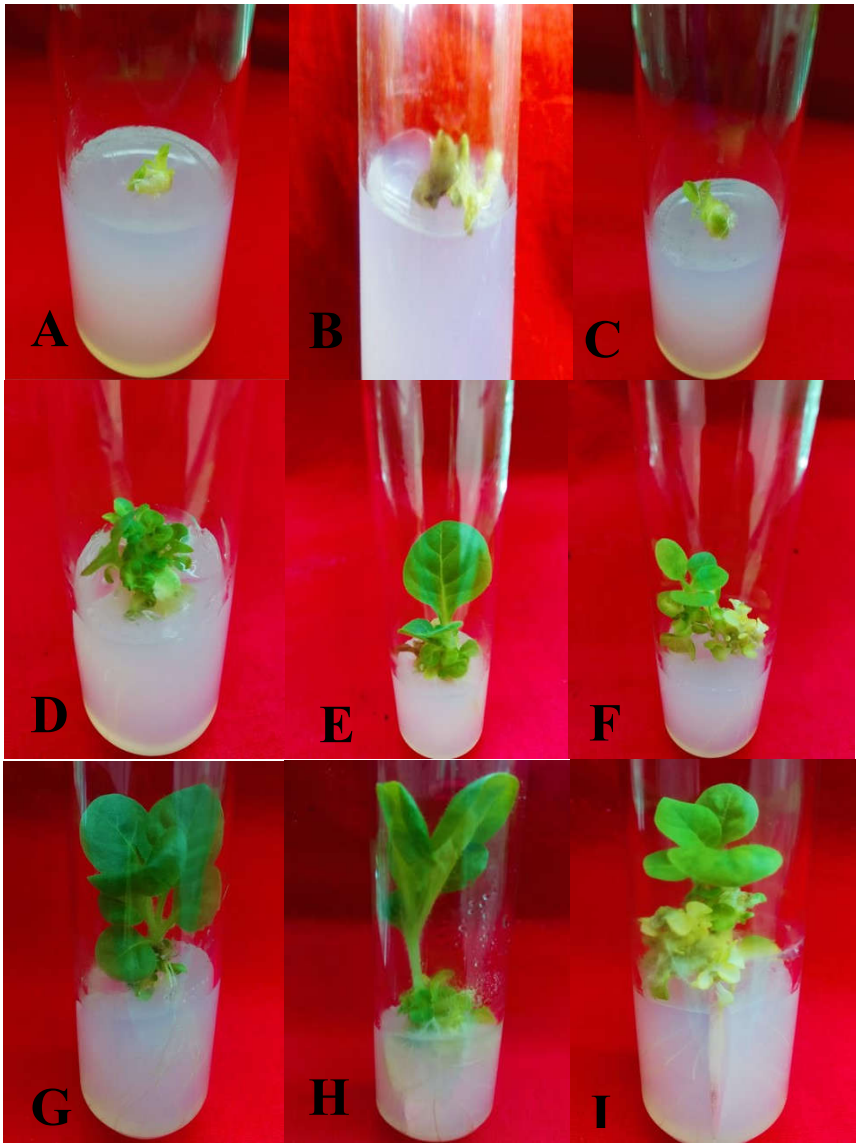

Fig. 2. Callus induction, shoot regeneration and root formation of *in vitro* derived explants. Callus induction from- (A) Cotyledon (B) Hypocotyl and (C) Leaf explants. Multiple shoots were regenerated from- (D) Cotyledon (E) Hypocotyl and (F) Leaf derived explants. *In vitro* rooting of (G) Cotyledon (H) Hypocotyl and (I) Leaf explants regenerated shoots

4. CONCLUSION

In the present study, we have successfully developed a simple, efficient and reproducible regeneration protocol from three explants-cotyledon, hypocotyl and leaf of tobacco (*N. tabacum* L.). Leaf explants is better and suitable compared to the others two explants (cotyledon and hypocotyl) in aspect of regeneration efficiency. Leaf explants of *N. tabacum* will be suitable for *Agrobacterium*-mediated genetic transformation for gene function analysis and study. Our developed regeneration protocol information will be helpful for further genetic transformation, gene function study in tobacco as well as others economically important crop plants.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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