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In vitro regeneration of *Nicotiana tabacum* L. from callus culture

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Abstract
Tobacco (*Nicotiana tabacum* L.) is a model plant in the field of tissue culture, plant regeneration and plant transgenic. The present study reveals the procedure for the regeneration of tobacco plants by organogenic mode. 3rd to 4th node young tobacco leaves were used as explants in this experiment. The callus were formed after leaves cultured onto tobacco regeneration medium supplemented with MS+ 0.1 mg/l NAA+1.0mg/l BAP the percentage of callus produced at this concentration was 55% with a mean size 3.80 cm and mean weight of 5.30 g respectively. The highest numbers of shoots per explants were obtained on the media containing MS+1.0 mg/l BAP +1.0 mg/l NAA which produced shoots per explants.

Keywords: BAP, callus, *In vitro*, NAA, *Nicotiana tabacum*, organogenesis, shoot regeneration

1. Introduction
Tobacco (*Nicotiana tabacum* L.) 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. Tobacco earns foreign currency which occupies 4th position after jute, sugarcane, and tea. Tobacco has been used as a model crop plant for *in vitro* studies on regeneration, since the classical studies of Skoog and Miller (1957) [21]. Totipotency was first demonstrated with *Nicotiana tabacum* by regeneration of mature plants from single cells (Vasil and Hildebrandt, 1965) [65]. Variability of regenerates has been obtained from tobacco tissue culture (Nikova and Zagorzka, 1984) [13]. Advanced quality hybrids and asymmetrical hybrids through protoplast fusion (Kortash and Kanevsku, 1987) [11], resistance to herbicide (Freyssinet, 1986) [7], obtaining TMV mosaic free plantlets through tobacco callus culture (Sanger *et al.,* 1986) [19], overcoming cross incompatibility and obtaining male sterile forms (Nikova *et al.,* 1988) [14], demonstration of gene transformation (Zhang *et al.,* 1998) [24] and determining effect of antibodies on the *in vitro* growth response (Silva *et al.,* 2003) [20] 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 callus genesis and plantlet regeneration using leaf disc to observe the callus induction potentiality in five tobacco cultivars. The regeneration of whole organisms depends upon the concept that all plant cells can, given the correct stimuli, express the total genetic potential of the parent plant, so this practice involves the culture environment, culture media, and growth regulators and so on. Both chemical and physical, of the plant cells have to meet by the culture vessel, the growth medium and the external environment (light, temperature, etc.). Culture media used for the *in vitro* cultivation of plant cells are composed of three basic components: (1) essential elements, or mineral ions, supplied as a complex mixture of salts; (2) an organic supplement supplying vitamins and/or amino acid; (3) a source of fixed carbon; usually supplied as the sugar sucrose. Date back 1962, MS medium, which was invented by Murashige and Skoog [Murashige, T. and F. Skoog 1962] [12], is an extremely widely used medium and forms the basis for many other media formulations. This classical medium was also used in this experiment. Plant growth regulators, divided into 5 classes that are auxins, cytokinins, gibberellins, abscisic acid, and ethylene are the critical media components. And several different culture types most commonly used in plant transformation studies such as Callus, Cell-suspension cultures,

protoplasts, root cultures, shoot tip and meristem culture, embryo culture and microspore culture. Tobacco was found somewhere in the American continent, but how and when it was first discovered is unknown. What is certain is that tobacco smoking was practiced among the early Mayas. After first cultivated in European country outside of the Americas in 1500s by Portuguese, tobacco plants were spread quickly. Not waiting for long, Portuguese introduced smoking into India, Eastern Asia and Japan in 1699. Since that, tobacco was planted widely. In 1997, the acreage of tobacco in China was up to 2.16 million hectare (Zhao Jianying 2003) [25], 42% of that in the world. Tobacco is one of the most important economic crop, which has been considerate to *fruit fly* of plant kingdom because it becomes to a classical mode plant that could be cultured *in vitro* and gain regenerated transformation plant easily (Jiao Zhen, Qin Guangyong, Huo Yuping 2003) [9].

2. Material and Method

2.1 Sterilization and seed germination

The tobacco seed were washed under running tap water for 10 min to remove surface adhering contaminants. Followed by surfactant treatment by dipping in 0.1% $HgCl_2$ for 10 min. The material was then thoroughly washed 2 to 3 times double distilled water. Then the seed were allowed to grow on standard growth media under pathogen-free conditions by supplying 16/8 h (light/dark) at room temperature (25±2 °C).

2.2 Preparation of growth regulator for callus induction and shoot regeneration

Callus induction and shoots regeneration from callus was obtained by using MS medium supplemented with plant growth regulators at different concentrations of NAA 0.1, 0.5, 1.0, 1.5 mg/l and 1.0 mg/l BAP. The shoots were regenerated from callus on MS media containing BAP 0.1, 0.5, 1.0, 1.5 mg/l and 1.0 mg/l NAA. The pH of the media was adjusted to pH 5.7 ± 1 prior to autoclaving for 20 min at 121 °C. After autoclaving, the MS media was allowed to cool at room temperature. Then, 15 ml medium was poured into petri plate. All culture media were maintained at room temperature for growth. The culture media was monitored for shoot production and callus fresh weight per explant at weekly intervals throughout 4 weeks period of time**.** *In vitro* Rooting The developed shoots were cut (1to2 in length) and inoculated on to the Tobacco rooting medium with 30% sucrose and without any grow the hormones. Young rooted plants were taken out of the culture tubes, washed with distilled water and planted in pots containing Hoagland media. The plants were watered once in two days initially, then once in a day after eight to ten days.

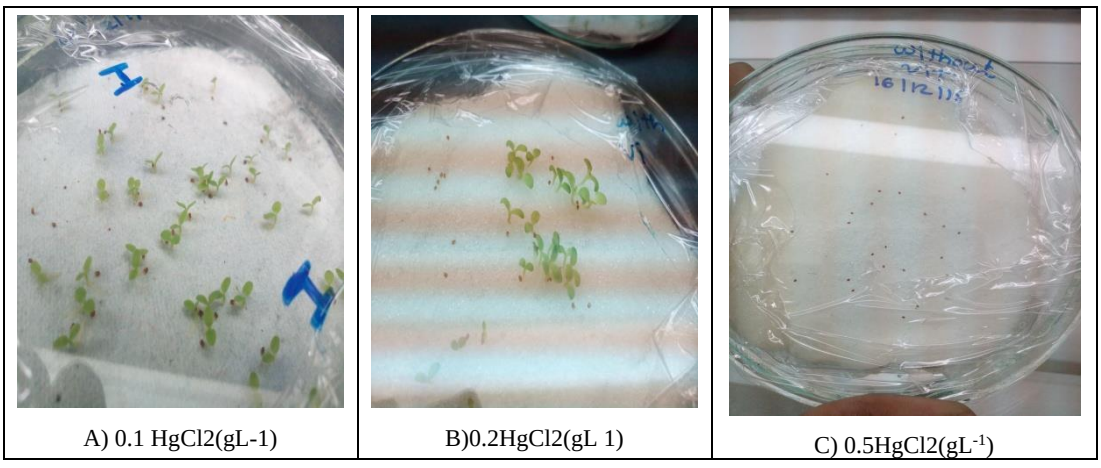
3. Result

3.1. Effects of Mercuric Chloride ($HgCl_2$) on Survival of Tobacco Seeds

Table No.1 shows the % of seeds survived when washed with various concentrations of HgCl2. The maximum number of seeds germinated was obtained at 0.1 and 0.2 g/l of HgCl2 showed 85 and 70% respectively. However, seeds washed with HgCl2 at 0.5 g/l were not survived suggesting at higher concentrations of HgCl2 was too toxic to the seeds. Fig.1. supported Table.1 showing the total number of seeds placed on MS media. It was 25 seeds per petri dishes with three replicates for each treatment. This observation was in agreement with the findings of Mohammed *et al*., that seed washed with 0.05gL-1 of $HgCl_2$ showed a good growth. At 0.05 gL-1 of $HgCl_2$the seed coat is free from contaminants, without killing the seeds. There are many scientific finding of surface sterilization in tobacco tissue culture using $HgCl_2$.

Table 1: The effects of different concentrations of $HgCl_2$ on seeds germination. Concentrations above 0.1 HgCl2(g/l) inhibit seeds germination

Concentrations of $HgCl_2$ (gL-1)	Time (min.)	Seed germination (%)
0.1	3	85
0.2	3	70
0.5	3	00



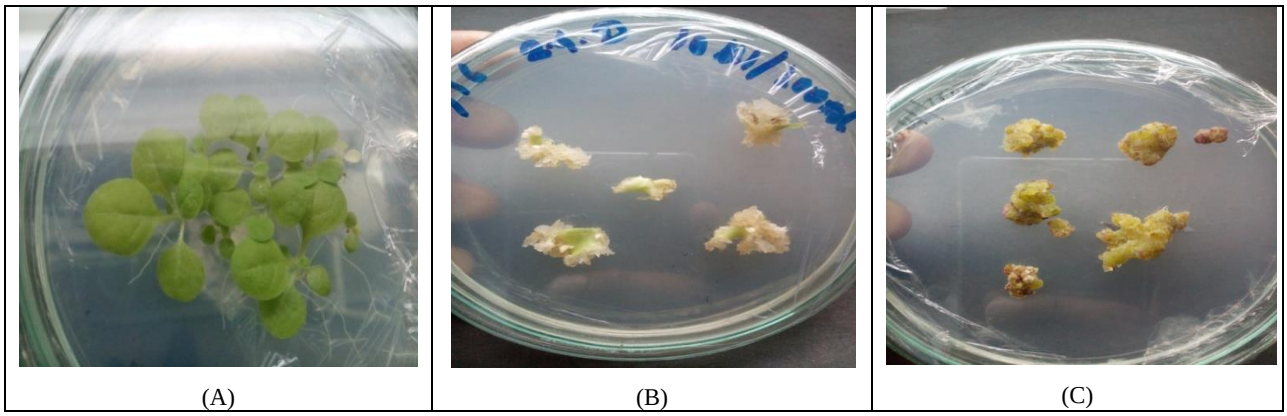
A) 0.1 HgCl2(gL-1) B)0.2HgCl2(gL 1) C) 0.5HgCl2(gL^{-1})

Fig 1: The growth of tobacco seeds after treated with various concentrations of $HgCl_2$; (A) the seeds still showing some growth at 0.1 g/l of (HgCl2).The concentrations of Mercuric Chloride at 0.1, 0.2, 0.5 g/l, on plates B and C respectively showing no seeds germination after two weeks in culture.

3.2 Effect of Combination Concentration of Auxins and Cytokinins on the Mean Callus Weight from Leaf Explant

Callus induction is significantly depending on explants type. Callus was induced by using leaf explants from *in vitro* grown plants during four weeks on MS media supplemented with various concentrations of plant growth regulators. Induction in monocots callus requires long time for its initiation. Figure 2 shows the highest callus size, callus weight and percentage of callus produced from explants were observed at MS+ 0.1 mg/l NAA+1.0mg/l BAP followed by variable callus

induction response on the other combination used as shown in Table No 2. The percentage of callus produced at this concentration was 55% with a mean size 3.80 cm and mean weight of 5.30 g respectively.



(A) (B) (C)

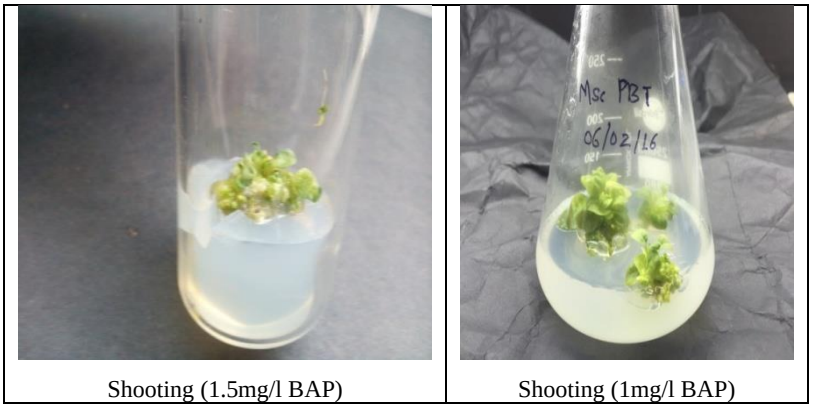
Fig 2: Callus formation stages - A) Leaf sample B) Callus Initiation C) Callus Induction

Table 2: The percent callus formation, mean size, mean weight

Concentration Medium+ Hormones	Callus%	Mean size (cm)	Mean weight(g)	Callus texture
Control	10	1.18	2.13	Soft and light yellow
MS+ 0.1 mg/l NAA+1.0mg/l BAP	55	3.80	5.30	Hard and light green
MS+ 0.5 mg/l NAA+1.0mg/l BAP	42	3.12	4.00	Compact greenish
MS+1.0 mg/l NAA+1.0mg/l BAP	6.89	1.07	1.80	Soft and yellowish
MS+1.5 mg/l NAA +1.0mg/l BAP	0.30	0.55	0.74	Soft and light yellow

3.3 Effect of Combination Concentration of Auxins and Cytokinins on the Shoot Formation from callus

BAP and NAA were used to investigate the number of shoots induction from callus culture. The effects of plant growth regulators on shoots proliferation were examined using callus. After four weeks of culture, the numbers of shoots per explants were recorded. Previous study has shown that BAP was one of the most effective among the other cytokinins for inducing shoot development. Figure 3 shows that the highest numbers of shoots per explants were obtained on the media containing MS+1.0 mg/l BAP +1.0 mg/l NAA which produced shoots per explants. the lowest number of shoots per explants were obtained when MS+ 0.5mg/l BAP +1.0mg/l NAA, while 2.0 mgL-1 NAA MS+ 0.1 mg/l BAP +1.0mg/l NAA resulted in no shoot formation at all.



Shooting (1.5mg/l BAP) Shooting (1mg/l BAP)

Fig 3: The effects of both BAP and NAA on shoot proliferation from callus culture

Table 3: The effect of shoot formation on tobacco plant a

Concentration Medium+ Hormones	No of Shoot after callus
Control	00
MS+ 0.1mg/l BAP +1.0mg/l NAA	00
MS+ 0.5mg/l BAP +1.0mg/l NAA	1
MS+1.0 mg/l BAP +1.0mg/l NAA	2.1
MS+1.5 mg/l BAP +1.0mg/l NAA	1.5

3.4 *In vitro* Root formation

The tobacco roots developing ability was shown on hormones free MS media.

Fig 4: Rooting without any growth regulators

4. Hardening

The *in vitro* grown plants when treated with 1% Bavistin fungicide solution had shown good fungal resistance. On transferring to sterilized potting mixture (vermiculite: soil mixture) in pot and irrigated with Hoagland solution the regenerated plants had developed proper shoot and root system. The complete regenerated plant of both cultivars were then transferred to green house.

Fig 5: Tobacco plant in pot

4. Conclusions

In the presence study we observed that the highest percentage of callus mean size and mean weight observed with MS+ 0.1 mg/l NAA+1.0mg/l BAP with 55%, 3.80cm, 5.30g respectively also the highest number of shoots The highest numbers of shoots per explants were obtained on the media containing MS+1.0 mg/l BAP +1.0 mg/l NAA which produced shoots per explants

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