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BAP and IBA pulsing for *in vitro* multiplication of banana cultivars through shoot-tip culture

M. H. Ferdous[a], A. A. Masum Billah[b], H. Mehraj[c], T. Taufique[d] and A. F. M. Jamal Uddin[d,*]

[a]*Dept. of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University, Dhaka-1207, Bangladesh*
[b]*Dept. of Agriculture Extension, Ministry of Agriculture, Iswardi, Pabna, Bangladesh*
[c]*The United Graduate School of Agricultural Sciences, Ehime University, 3-5-7 Tarami, Matsumaya, Ehime 790-8556, Japan*
[d]*Dept. of Horticulture, Sher-e-Bangla Agricultural University, Dhaka-1207, Bangladesh*

ASTRACT

The study was undertaken with a view to establish a protocol for in vitro plant regeneration from shoot tip explants of banana. Different concentrations of BAP (0.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 mg/l) and IBA (0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) was used in MS medium to assess the influence on in vitro shoot regeneration and subsequent root formation of Amritasagar and Sabri banana cultivars. Maximum single shoot formation (50.0% and 30.0%), number of single shoot (3.50 and 2.00 in), longest shoot (2.64 cm and 2.16 cm) were found from 0.5 mg/l BAP while maximum number of roots (3.83 and 2.50) and longest root (3.60 cm and 3.10 cm) was found from 0.3 mg/l IBA in Amritasagar and Sabri respectively. The survival of the plantlets of both cultivars was more than 82% under ex vitro condition. 0.5 mg/l BAP and 0.3 mg/l IBA can be used with MS media for shoot and root formation of Amritasagar and Sabri banana cultivars through shoot tip culture.

Keywords: *Banana, growth regulators, in vitro shoot-tip culture and plantlets regeneration*

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I. Introduction

Banana (*Musa* spp.) belongs to Musaceae family. It is clonally propagated and attacked by wide range of diseases and pests (Cronauer and Krikorian, 1983) which affect yield. Disease free plant material should be produced to minimize this problem which can be possible by meristem culture because pathogen concentration gradually decreases in shoot tips and considered to be absent or less in meristem. Tissue culture plants have been reported to produce 39% higher yield than plants from sword suckers (Pradeep *et al*., 1992) likely performed better than in banana (Faisal *et al*., 1998). Shoot apex, nodal segments and root segments were successfully used for callus induction and regeneration (Jatoi *et al.*, 2001) and cytokinin helps in shoot multiplication (Cronauer and Krikorian, 1984a).

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Growth regulators namely auxin, cytokinin, gibberellin and abscisic acid like kinetin, indole-3-acetic acid, benzylaminopurine etc were used for the *in vitro* regeneration of various plants (Ali *et al.*, 2014 and 2015; Momena *et al.*, 2014). Cytokinins such as benzylaminopurine (BAP) are known to reduce apical meristem dominance and induce both axillary and adventitious shoots formation from meristematic explants in banana (Madhulatha *et al.*, 2004). Effectiveness of BAP over other cytokinins in inducing multiplication of shoot tip cultures has been reported in different banana cultivars (Buah *et al.*, 2010; Farahani *et al.*, 2008; Rahman *et al.*, 2006; Resmi and Nair, 2007). The most established banana shoot-tip culture system was achieved by using BAP as a supplement to basal media (Murashige and Skoog, 1962) and its effect in stimulating growth of axillary and adventitious buds also foliar development of shoot tip cultures (Buah *et al.*, 2010). Considering the constraints behind disease free planting materials and potential of tissue culture through shoot-tip and or meristem, the study was undertaken to assess the suitable concentration of BAP for *in vitro* shoot proliferation and root induction of two banana cultivars, namely, cv. Amritasagar and cv. Sabri, for plantlets regeneration.

II. Materials and Methods

Laboratory and period of the experiment: The study was carried out at the laboratory of Proshika Tissue Culture Centre Trust, Manikgonj, Bangladesh from the April, 2008 to November, 2008.

Genetic materials: Banana cv. Amritasagar (*Musa sapientum*, Genotype AAA) and Sabri (*Musa sapientum*, Genotype AAA) were used in the experiment.

Concentrations of BAP and IBA: Different concentrations of BAP (0.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 mg/l) on shoot multiplication and IBA (0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 mg/l) for rooting of two cultivars of banana was studied.

Culture media for *in vitro*: Both for shoot regeneration and rooting of multiplied shoots MS medium (Murashige and Skoog, 1962) was used with different vitamins and hormonal supplementation. Hormones were added separately to different media according to the requirements. For the preparation of media, stock solutions were prepared at the beginning and stored at 4±1^{0}C temperature. The respective media were prepared from the stock solutions. The culture tubes containing media, beakers, pipettes, measuring cylinder, metal instruments were autoclaved at 15 psi pressure at 121^{0}C for 20 minutes. The medium was then cooled at room temperature before use. Laminar Airflow Cabinet was usually sterilized by switching on the UV light of the cabinet for 30 minutes and wiping the working surface with 70% ethyl alcohol for 30 minutes before starting the transfer work.

Sample collection and preparation for *in vitro* culture: Meristem was collected from developing suckers from field for both cultivars. The suckers were washed thoroughly under running tap water. The roots and outer tissues of the suckers were removed with the help of a sharp knife. A number of outer leaves were removed until the shoot measured about 1.5-2.0 cm in length and 1.0 cm width at the base. The prepared explants were taken into a conical flask and were washed with distilled water containing 1% savlon and 3-4 drops of Tween-80 for 20 minutes to remove dusty substance and followed by successive 3 times washing with distilled water to make the materials free from savlon and Tween-80. Subsequently the materials were transferred to running Laminar Airflow Cabinet.

Surface sterilization: The surface sterilization of explants was carried out in 0.1% $HgCl_2$ for different periods (10, 12, 14, 16 minutes). Then the materials were washed 3-5 times with distilled water to remove all traces of $HgCl_2$.

Inoculation of plant materials: The shoot tip explants of about 0.5 cm long with 3-4 leaf primordial were prepared. The individual shoot tip was directly inoculated to each culture vessel and covered with plastic cap. After that the caps were sealed with parafilm.

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Transfer to growth chamber: The culture vessels were transferred to growth room and were allowed to grow in controlled environment (25±2^{0}C temperature, 16-hour light period and 2000 lux light intensity) for the growth and development of the cultures.

Sub-culturing: For sub-culturing, the entire samples of *in vitro* shoots were cut into small pieces so that each piece would contain about one shoot. Leaf and blackish or browned basal tissues were removed to expose the meristem. Each piece was inoculated into a similar fresh medium. It was practiced at the interval of every one month. *In vitro* proliferated micro shoots were separated and each of the micro shoots was placed on culture medium.

III. Results and Discussion

Effect of different strength of $HgCl_2$

After 10 days of sterilization and inoculation, it was observed that when the explants were treated with 0.1% $HgCl_2$ for 14 minutes, 100% shoot tip explants of Amritasagar and Sabri were found free from any contamination (Table 01). On the other hand, the percentage of contaminated explants increased at 20 days after sterilization and inoculation because there were endogenous bacteria in the meristem of explants that expressed later. When $HgCl_2$ was used for short durations (10 and 12 minutes), the treatments failed to kill the microorganisms attached to the surface of field grown explants. Among the periods of treatment, the highest percent of explants survived at 14 minutes (Table 01).

Treatments for more than 14 minutes duration survival percentage was decreased and tissue became damage. So, concentrations of disinfectants and soaking time are adjusted according to the resistance of the explants to sterilants. Microbes like bacteria, fungi and virus are the most common contaminants observed in cultures. To overcome this sterilizing agents with different concentrations are generally used like 0.25% sodium hypochlorite (Vessey and Revera, 1981) chlorine saturated water for 15-20 minutes (Dore *et al*., 1983), 0.05% sodium hypochlorite (Cronauer and Krikarion, 1984a).

Table 01. Standardization of $HgCl_2$ treatment period for surface sterilization of shoot tips of Amritasagar and Sabri

Cultivars	Treatment period of 0.1% $HgCl_2$ (min.)	No. of explants cultured	% of explants contaminated after		% of explants survived after
			10 days	20 days	20 days
Amritasagar	10	10	60	80	20
Sabri	10	10	50	70	30
Amritasagar	12	10	30	40	60
Sabri	12	10	30	40	60
Amritasagar	14	10	-	10	90
Sabri	14	10	-	10	90
Amritasagar	16	10	-	20	80
Sabri	16	10	-	30	70

Explants were surface sterilized with 0.1% $HgCl_2$. Contamination free culture with higher surviving ability was achieved by treating the explants with 0.1% $HgCl_2$ for 14 minutes. It indicated that the duration of soaking of explants and the concentrations of the disinfectants were important factors to sterilize the explants.

Different concentrations of BAP on shoot multiplication

Relative colour changes of explants: After inoculation to the culture media, the shoot tips showed creamy white appearance at first sight and gradually became brown to light green, green and finally dark green on MS medium supplemented with different concentrations of BAP (Plate 01 and 02) and colour changing observed regularly.

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The explants became dark green at 3.0 mg/l and 5.0 mg/l at 10 DAI (days after inoculation) but at 1.0 mg/l, 2.0 mg/l, 4.0 mg/l, 6.0 mg/l and 7.0 mg/l BAP the inoculated explants became green and light green in control while all explants treated with BAP turned dark green in colour except in control i.e., 0.0 mg/l BAP at 20 DAI in Amritasagar cultivar (Table 02). In Sabri cultivar, after 10 days, the explant colour was green for 3.0 mg/l, 6.0 mg/l and 7.0 mg/l BAP concentrations while light green for 1.0 mg/l, 2.0 mg/l, 4.0 mg/l and 5.0 mg/l BAP concentrations but brown in control (Table 02). On the other hand, after 20 days, all the explants turned dark green for all concentrations of BAP except in control and 1.0 mg/l BAP (Table 02). In control, the colour was light green in Sabri and green in Amritasagar after 20 DAI (Rabbani *et al.*, 1996). Therefore, it was clear that BAP was essential for early greening of the banana cultivars. Colour changes of inoculated explants showed clear variation in the same treatment for different varieties. It was probably due to genotype variation. Presence of cytokinin in the medium was essential for greening of banana shoot tip explants (Ranjan *et al.*, 2001).

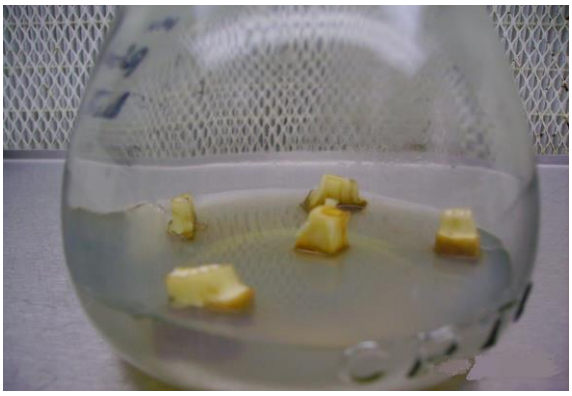

Plate 01. A just inoculated shoot tip isolated from a sucker showed creamy white colour

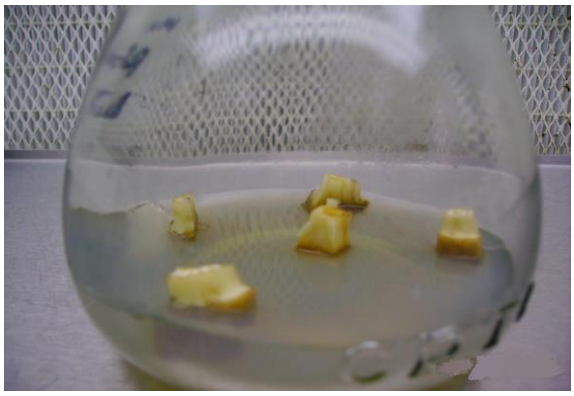

Plate 02. Inoculated shoot tip became dark green in colour

Table 02. Effect of different concentrations of BAP on relative colour changes of banana cv. Amritasagar and Sabri

Treatments	Banana cultivars			
	Amritasagar		Sabri	
Conc. of BAP (mg/l)	10 days	20 days	10 days	20 days
0.0	+	+ +	B	+
1.0	+ +	+ + +	+	+ +
2.0	+ +	+ + +	+	+ + +
3.0	+ + +	+ + +	+ +	+ + +
4.0	+ +	+ + +	+	+ + +
5.0	+ + +	+ + +	+	+ + +
6.0	+ +	+ + +	+ +	+ + +
7.0	+ +	+ + +	+ +	+ + +

B indicates Brown; + indicates Light Green, ++ indicates Green, +++ indicates Dark Green

Tuber like structure developed from meristem explants: The cultured meristem formed hard meristematic tuber like structure in regeneration media containing different concentrations of BAP. In Amritasagar and Sabri, maximum rate of forming tuber like structure were 60.0% and 40.0% respectively on MS medium with 5.0 mg/l BAP while minimum 20.0% and 10.0% in Amritasagar and Sabri respectively on MS medium without any hormone. Therefore, Amritasagar showed better performance in formation of tuber like structure over Sabri. Some tuber like structures formed from the base of the shoot during shoot multiplication (Habib, 1994). These structures of banana are very good for germplasm preservation and can be utilized for further multiplication and subsequently mass proliferation of ideal plantlets for commercial exploitation.

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Survivability: Maximum survivability was found in MS medium containing 5.0 mg/l BAP (90.0% in Amritasagar and 80.0 % in Sabri) and minimum in 0.0 mg/l BAP (50.0% in Amritasagar and 40.0% in Sabri) (Table 03). However Amritasagar showed better survivability over Sabri for all the treatments.

Regeneration of single shoot from meristem explants: Maximum single shoot from meristem was regenerated at 5.0 mg/l BAP (50.0%) whereas minimum from 1.0 mg/l BAP (20.0%) in Amritasagar cultivar and maximum single shoot from meristem was produced at 5.0 mg/l BAP (30.0%) whereas minimum at 1.0 mg/l BAP (10.0%) in Sabri while single shoot was not regenerated in control (Table 03). It was observed that single shoot regeneration was lower in Sabri compared to Amritasagar and the average time required for single shoot regeneration was higher than Amritasagar (Table 03). BAP was required for the single shoot regeneration (Rabbani *et al.*, 1996). The results indicated that the percentage of single shoot regeneration increased with the increase of BAP concentration up to 5.0 mg/l and there after declined.

Days to single shoot development: Early single shoot was developed at 5.0 mg/l BAP (12.0 days by Amritasagar and 14.0 days by Sabri) (Table 03). Time requirements for single shoot formation to be 10-15 days (Rehana, 1999) which was close to the present investigation.

Table 03. Response of cv. Amritasagar and Sabri banana cultivars to different concentrations of BAP at 20 DAI from meristem *in vitro*

Concentration of BAP (mg/l)	Banana cultivars							
	Amritasagar				**Sabri**			
	Tuber like structure (%)	Survivability (%)	Single shoot from meristem (%)	Days to single shoot development	Tuber like structure (%)	Survivability (%)	Single shoot from meristem (%)	Days to single shoot development
0.0	20.0	50.0	-	-	10.0	40.0	-	-
1.0	30.0	70.0	20.0	17.0	30.0	50.0	10.0	20.0
2.0	30.0	70.0	30.0	16.0	20.0	70.0	20.0	19.0
3.0	40.0	80.0	40.0	15.0	30.0	70.0	20.0	17.0
4.0	50.0	80.0	40.0	13.0	30.0	60.0	30.0	15.0
5.0	60.0	90.0	50.0	12.0	40.0	80.0	30.0	14.0
6.0	40.0	70.0	40.0	14.0	30.0	70.0	20.0	15.0
7.0	30.0	60.0	30.0	15.0	20.0	50.0	20.0	15.0

*Ten explants were inoculated/treatment

Number of shoots per explant: Maximum number of shoots proliferation per explant was found from 5.0 mg/l BAP (3.50 in Amritasagar and 2.00 in Sabri) at 30 DAI while both cultivars showed no response in 0.00 mg/l BAP (Table 04). The multiple shoot regeneration due the application of 0.5 mg/l BAP treatments was presented in Plate 03 and 04. The number of shoots increased with the increase of BAP concentration up to 5.0 mg/l and then decreased. 5.0 mg/l BAP showed the best performance and Amritasagar was better variety than Sabri. BAP at the rate of 5.0 mg/l produced the highest number of shoots in Amritasagar and Mehersagar (Rabbani *et al.*, 1996). Banana (AAA) meristem tip generated maximum number of shoots on MS medium supplemented with 6 mg/l BAP in the cultivar Amritasagar (Khanam *et al.*, 1996; Domingues *et al.*, 1995).

Shoot length: Shoot length also influenced by cultivars and different concentrations of BAP Data at 10, 20 and 30 DAI (Table 05). Longest shoot was found from 5.0 mg/l BAP in both cultivars but the cultivar Amritasagar produced longer shoot (2.64 cm) compared to Sabri (2.16) at 30 DAI (Table 04) whereas shoots were not produced when the BAP were not used on the culture media. 5.0 mg/l BAP was provided the longest shoot (Rabbani *et al.*, 1996; Khanam *et al.*, 1996.) and Amritasagar showed better performance over Sabri in case of shoot length. Amritasagar showed better performance over Sabri in case of shoot length.

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Table 04. Combined effect of cultivars and concentrations of BAP on number of shoots per explants and length of shoots

Cultivars	Conc. of BAP (mg/l)	Number of shoots per explant at different days after inoculation			Shoot length (cm) at different days after inoculation		
		10 days	20 days	30 days	10 days	20 days	30 days
Amritasagar	0.0	0.00	0.00	0.00	0.00	0.00	0.00
	1.0	0.00	0.33	0.60	0.00	0.09	0.86
	2.0	0.30	0.62	1.65	0.04	0.71	1.82
	3.0	0.65	1.33	2.25	0.08	1.30	2.35
	4.0	0.67	1.33	2.33	0.09	1.35	2.47
	5.0	1.00	1.72	3.50	0.14	1.36	2.64
	6.0	0.36	0.71	1.33	0.06	0.91	2.13
	7.0	0.30	0.37	1.00	0.00	0.42	0.96
Sabri	0.0	0.00	0.00	0.00	0.00	0.00	0.00
	1.0	0.00	0.00	0.33	0.00	0.00	0.33
	2.0	0.00	0.32	1.30	0.00	0.30	1.15
	3.0	0.00	0.65	1.32	0.00	0.70	1.60
	4.0	0.00	0.68	1.35	0.00	0.73	1.82
	5.0	0.00	1.00	2.00	0.00	0.90	2.16
	6.0	0.00	0.69	1.00	0.00	0.26	1.54
	7.0	0.00	0.67	0.68	0.00	0.23	0.95

Plate 03. Multiple shoot of Amritasagar produced on MS medium containing 5.0 mg/l BAP at 30 days after inoculation

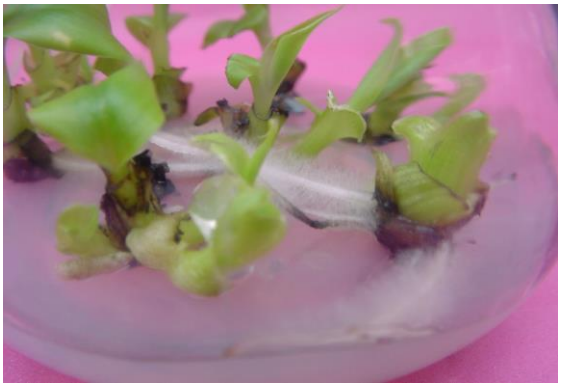

Plate 04. Multiple shoot of Sabri produced on MS medium containing 5.0 mg/l BAP at 30 days after inoculation

Number of leaves per plantlet: Among the concentrations of BAP, 5.0 mg/l showed maximum number of leaves in both the cultivars at different DAI. The cultivar Amritasagar produced 4.11 leaves per plantlet in 5.0 mg/l BAP at 30 DAI while Sabri produced 2.67 leaves per plantlet at the same level of BAP and DAI (Table 05). Leaf number per plantlet increased with the increase of BAP concentration up to 5.0 mg/l and then decreased in both the cultivars. Rabbani *et al.* (1996) also found that 5.0 mg/l BAP concentration was the best for number of leaves per plantlets.

Leaf length: Length of leaves produced per plantlet varied on MS medium supplemented with different cultivars and concentrations of BAP were used. The response of cultivars on leaf increment at different DAI was found significant. Among the concentrations of BAP, 5.0 mg/l showed the longest and 1.0 mg/l showed the shortest leaf in both the cultivars at different DAI. Amritasagar produced 2.29cm leaf at 5.0 mg/l BAP at 30 DAI while Sabri produced 1.86 cm leaf (Table 05). These results were in partial support of Rabbani *et al.* (1996) who obtained longest leaf with 5.0 mg/l each of BAP and Kinetin. BAP used on the culture media @ 0.5 mg/l provided the longest leaf (Khanam *et al.*, 1996).

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Table 05. Combined effect of cultivars and concentrations of BAP on number of leaves and leaf length

Cultivars	Conc. of BAP (mg/l)	Number of leaves per plantlets at different days after inoculation			Leaf length (cm) at different days after inoculation		
		10 days	20 days	30 days	10 days	20 days	30 days
Amritasagar	0.0	0.00	0.00	0.00	0.00	0.00	0.00
	1.0	0.00	0.67	1.17	0.00	0.04	0.72
	2.0	0.50	1.17	2.33	0.03	0.55	1.48
	3.0	1.00	2.17	3.50	0.07	1.05	1.85
	4.0	1.00	2.33	3.50	0.07	1.11	1.91
	5.0	1.50	2.50	4.11	0.11	1.21	2.29
	6.0	0.67	1.00	2.17	0.05	0.60	1.72
	7.0	0.00	0.50	1.50	0.00	0.12	0.78
Sabri	0.0	0.00	0.00	0.00	0.00	0.00	0.00
	1.0	0.00	0.00	0.50	0.00	0.00	0.53
	2.0	0.00	0.50	2.00	0.00	0.14	0.93
	3.0	0.00	1.00	2.00	0.00	0.41	1.29
	4.0	0.00	1.19	2.17	0.00	0.56	1.48
	5.0	0.00	1.67	2.67	0.00	0.72	1.86
	6.0	0.00	1.17	1.50	0.00	0.19	1.46
	7.0	0.00	1.15	1.00	0.00	0.13	0.71

Different concentration of IBA on root induction

Number of roots per plantlet: *In vitro* root induction for Amritasagar and Sabri cultivar was shown in Plate 5and 6 at 3.0 mg/l IBA after 30 DAI. Maximum number of roots per plantlets was found from 3.0 mg/l IBA in both cultivars (4.10 in Amritasagar and 3.63 in Sabri) at 30 DAI whereas no response was observed in control (Table 06). 3.0 mg/l IBA was the best treatment among all concentrations of BAP for this parameter (Habib, 1994; Rabbani *et al.*, 1996; Raut and Lokhande, 1989). Amritasagar produced more number of roots than Sabri.

Root length: Longest root was found from 3.0 mg/l IBA (3.60 cm in Amritasagar and 3.10 cm in Sabri at 30 DAI) and 1.0 mg/l produced the shortest root in both the cultivars at different DAI whereas no response was observed in control. 3.0 mg/l IBA was the best treatment among all the concentrations for this parameter (Bhaskar *et al.*, 1993).

Plate 05. *In vitro* root induction of Amritasagar at 3.0 mg/l IBA after 30 DAI

Plate 06. *In vitro* root induction of Sabri at 3.0 mg/l IBA after 30 DAI

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Table 06. Combined effect of cultivars and concentrations of IBA on root number and root length

Cultivars	Conc. of IBA (mg/l)	Roots per plantlet at different days after inoculation			Root length (cm) at different days after inoculation		
		10 days	20 days	30 days	10 days	20 days	30 days
Amritasagar	0.0	0.0	0.0	0.00	0.00	0.00	0.00
	1.0	0.42	1.0	1.27	0.10	0.57	1.14
	2.0	1.60	2.92	3.21	0.45	1.20	2.30
	3.0	1.67	3.83	4.10	0.98	2.20	3.60
	4.0	1.74	2.60	3.72	0.90	1.55	2.72
	5.0	0.83	2.08	3.01	0.85	1.60	2.00
Sabri	0.0	0.0	0.0	0.00	0.00	0.00	0.00
	1.0	0.0	0.47	0.60	0.00	0.40	0.90
	2.0	0.42	1.25	2.42	0.25	0.76	2.07
	3.0	1.08	2.50	3.63	0.58	1.90	3.10
	4.0	0.93	1.75	2.42	0.47	1.20	1.72
	5.0	0.83	1.33	2.30	0.46	1.02	1.35

Ex vitro hardening of plantlets

The plantlets with well-developed roots were removed from the culture vessels without damaging the roots. The culture media was washed away from the roots with running tap water. After that, the plantlets were treated with fungicide (1.5-2 g/l Ridomil) for 20 minutes to prevent unwanted fungal or bacterial growth in the roots. These plantlets were transferred to small polythene bag filled with soil, sand and well decomposed cow dung (1:1:1) and kept in the hardening room for 5-8 days. Then the polythene bags containing plantlets were transferred to net house, where proper care was taken for growth and development of banana plantlets. After 15-20 days, maximum plantlets showed vigorous growth while some were less vigorous. These plantlets were transferred to the field conditions after 30 days where they grew under conditions of normal environment. The survival rate of plantlets was more than 82% for both the cultivars. 80% survival of plantlets was found under *ex vitro* condition (Azad and Amin, 1999) and similarly by Cronaur and Krokorian (1984a and 1984b).

IV. Conclusion

Application of 0.5 mg/l BAP and 0.3 mg/l IBA as growth supplements on MS culture media help to perform better for new plantlet regeneration capability through *In vitro* shoot-tip culture of Banana. BAP concentration 0.5 mg/l and IBA concentration of 0.3 mg/l was found best concentration for shoot proliferation and root elongation in both cultivars of banana. On the other hand, Application of more than 0.5 mg/l BAP and 0.3 mg/l IBA gradually decreases *In vitro* culture developments for all parameters as observed for shoot proliferation and root elongation. But for the commercial plantlet regeneration, Amritasagar was found better than Sabri cultivar with 0.5 mg/l BAP and 0.3 mg/l IBA for shoot regeneration and root formation respectively.

V. References

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Corresponding author email: jamal4@yahoo.com (Jamal Uddin, A. F. M.)
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Corresponding author email: jamal4@yahoo.com (Jamal Uddin, A. F. M.)
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