Eradication of Banana Bunchy Top Virus (BBTV) and Banana Mosaic Virus (BMV) from Infected Plant of Banana cv. Amritasagar Through Meristem Culture

M. H. Kabir SHIRAGI[1*], M. A. BAQUE[2] and K. M. NASIRUDDIN[3]

1:Department of Horticulture, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh
2:Department of Agronomy, Sher-e-Bangla Agricultural University, Dhaka-1207, Bangladesh
3:Department of Biotechnology, Bangladesh Agricultural University, Bangladesh

Abstract

An experiment on virus free in vitro banana plantlets production by the meristem cultured from virus infected four month old banana suckers cv. Amritasagar were used as explants. Four levels of BAP (0, 3, 4 and 5 mg/l), 5 levels of NAA (0.0, 1.0, 1.5, 2.0 and 2.5 mg/l) were used as treatment for shoot proliferation in experiment-I and Four levels of IBA (0, 1, 2 and 3 mg/l), 4 levels of NAA (0, 2, 3 and 4 mg/l) were used for root formation in experiment-II. ELISA test was conducted for the confirmation of virus eradication rate of regenerated plants and survival rate of regenerated plants in experiment-III. A significant variation was found among the treatments. Studies on the different concentrations of BAP + NAA on shoot proliferation and differentiation, reveals that 4 mg/l BAP + 1.5 mg/l NAA produced the greater number of shoots (4.58/explant), the longest shoot (3.56 cm) and the highest numbers of leaves (3.11/explant). On the contrary, plantlets were cultured on half strength of MS media in different levels of IBA and NAA.The highest number of vigorous roots (6.98/explant) and maximum root length (5 cm) were recorded from the treatment combination 2 mg/l IBA+2 mg/l NAA and 3 mg/l IBA+ 3 mg/l NAA, respectively. While less vigorous root was found in control treatments. The rate of virus free plant regeneration from banana bunchy top virus (BBTV) infected plants was 57.14% and from banana mosaic virus (BMV) infected sources was 64.28%. The highest plant survival rate 83.33% was recorded in a mixture of sand, soil and cow dung (1:1:1). On the other hand, 67.67% plant survival rate was found in a mixture of coir and soil (1:2). Eradication of banana viruses from infected plants by meristem cultured is a useful tactic that will be helpful for commercial exploitation and virus free plant production for use of farmer's level.
Key words: Plants growth regulators, meristem culture, shoot & root proliferation, ELISA and Amritasagar

Introduction

The banana and plantains (*Musa spp.*) belonging to the family Musaceae are one of the world's most important subsistence crops. In Bangladesh, banana is popular for its year round availability, abundant production as well as high acceptability to the consumers. It is a rich source of carbohydrate and also rich in some minerals, notably phosphorus, calcium and potassium. Banana is particularly rich in vitamin-C and also

*mhkshiragi@yahoo.com
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contains significant amounts of several other vitamins (INIBAP 1987). In addition, it has importance for tannin, latex and fiber production. Banana ranks first in terms of production and second in terms of area, among the fruit crops and so has commercial value in Bangladesh. It is comprising nearly 42% of the total fruit production of the country. It occupies an area of 43 thousand hectares of land with total production of 606 thousand metric tonnes with an average yield of 14.16 t/ha (BBS 2003). This yield is quite low compared to other banana growing countries of the world like Argentina (34 t/ha) and Costa Rica (33t/ha) (FAO 2002).

Many natural calamities, such as abiotic and biotic factors cause devastating losses in banana production. Some major problems are also caused by viruses. Virus diseases are serious, as insect vectors are abundant and there are many alternate hosts. It is affected by four known relatively well characterized viruses (Diekmann and Putter, 1996) namely, banana bunchy top virus (BBTV), banana streak virus (BSV), banana mosaic virus (BMV) and banana bract mosaic virus (BBrMV). Banana bunchy top and banana mosaic virus are the most important virus diseases of banana in this region. At present, there are no known banana varieties resistant to BBTV & BMV and no chemicals to control it (MAGEE 1927, DALE 1987). However, there are obvious advantages in ensuring that only virus free plants are used for propagation by meristem culture.

Tissue culture is now a standard practice in banana propagation. Conventional non-molecular control means such as quarantine, eradication and certified virus free stock have successfully been used to reduce crop losses caused by these viruses. But those were not effective enough for controlling BBTV and BMV. Recently, molecular genetic engineering techniques have become available and new approaches have been suggested to control banana virus diseases. Meristem culture offers an efficient method for rapid clonal propagation, production of virus free materials and germplasm preservation in plants (CRONAUER and KRIKORIAN, 1984, HWANG *et al*. 1994 and HELLOIT *et al*. 2002). As regards yield performance, tissue cultured plants have been reported to produce 39% higher yield than plants from sword suckers (PRADEEP *et al*. 1992). Under Bangladesh conditions, tissue culture derived plantlets of banana performed better than the conventional sword suckers (FAISAL *et al*. 1998).

Plant growth regulators are the essential part for *in vitro* regeneration of crop plants grown in any artificial medium. Generally, cytokinin helps in shoot proliferation and auxins helps in rooting of proliferated shoots. However, the requirement of cytokinin and auxins depends on the variety of banana and culture conditions (CRONAUER and KRIKORIAN 1984). To obtain virus and disease free healthy planting materials, development of a protocol for meristem culture of banana are of prime importance. Therefore, considering the above facts the present study was undertaken with a view to i) determine the appropriate hormone concentration for shoot proliferation and rooting, ii) standardize different hormonal concentration for banana meristem culture for virus free plantlet production and iii) evaluate BBTV and BMV resistance in the banana plants under green house and field conditions.

Materials and Methods

The experiment on virus free banana plantlets production by meristem culture and establishment of virus free *in vitro* plantlets into rooting media was conducted at the Biotechnology Laboratory, Department of Biotechnology, Bangladesh Agricultural University, Mymensingh, during September 2003 to March 2004. Banana cv. Amritasagar was used as planting materials. MURASHIGE and SKOOG (1962) medium supplemented with different phytohormones as per treatments were used as culture medium. For the preparation of media, different stock solutions e.g. Stock solution I (Macronutrients), Stock solution II (Micronutrients), Stock solution III (Iron source), Stock solution IV (Vitamins and amino acids), Stock solution V (Growth regulators), hormone stock solution VI were prepared at the beginning and stored at 4±1°C. The culture vessels with media, glassware and instruments were then autoclaved for sterilization at 1.16-kg/cm^2 pressure at 121°C for 20 minutes. The medium was then cooled at room temperature before use. Initially, the culture room was cleaned by gently washing all floors and walls with detergent or Lysol (germicide). This was followed by carefully wiping them with 70% ethyl alcohol. The transfer area was also sterilized twice a month by 70% ethyl alcohol. Laminar Airflow Cabinet was usually sterilized by switching on the cabinet and wiping the working surface with 70% ethyl alcohol for 30 minutes before starting the transfer work.

Culture techniques

Explant culture

The meristem was the starting material. It was obtained form developing infected suckers (about four months of age) of banana cv. Amritasagar grown under field conditions and was brought to the preparation room. 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 1.0 - 2.0 cm in length and 1.0 cm width at the base. Then the initial explant was prepared under stereomicroscope by removal of outer tissue of meristem with the help of sterile scalpel, which was about (5×5 mm) in size.

Surface sterilization of explants

The pale white tissue block (1.0×2.0 cm) containing meristem and rhizomatous base were taken in a beaker. Surface sterilization was done under Laminar Airflow Cabinet with 70% ethanol for one minute. After sterilization with 70% ethanol, the explants were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) and a few drops of Tween-20 for 15 minutes. After that, the explants were then rinsed three to four times with sterile dw. Finally, the explants were surface sterilized by renamycil capsule dissolved solution.

Culture explantation

The isolated and surface sterilized meristem tissue blocks were prepared carefully under (the stereomicroscope through maintaining) aseptic condition inside the laminar airflow cabinet, to use those as explants. The individual meristematic tissue was directly inoculated to each of the culture tube containing 20 ml of MS medium supplemented with different concentration of hormones required as per treatment, covered with plastic cap and were sealed with parafilm.

Incubation

The culture tubes were transferred to growth room and were allowed to grow in controlled environment. The temperature of the growth room was maintained with in 25 ±2°C by an air conditioner. A 16-hour light period was maintained with light intensity of 2000 lux of the growth and development of culture.

Browning of the explant

Some explants become black or brown in colour within 6-7 days after inoculation. To control blackening or browning after about one week the blackish or browning tissues on the explants were removed and the meristematic tissues were transferred to similar fresh medium. It was repeated each of 10 days interval for about one month to minimize further blackening or browning of the tissues.

Subculture or Transfer

Maintenance of proliferating shoots

Initial sub culturing was done when the explants had produced some shoots. For subculturing, in vitro shoot 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. Through subculturing, the numbers of shoots were increased.

Regeneration of plants from *in vitro* proliferated buds

In vitro proliferated micro shoots were separated and each of the micro-shoot was placed on culture medium, which supplemented with different concentration of hormone for shoot differentiation.

Root induction of regenerated shoots

When the shoots grown about 3-5 cm in length with 2-3 well developed leaves they were rescued aseptically from the culture vessels and were separated from each other and again cultured on freshly prepared medium containing different combinations of hormonal supplements for root induction. The produced shoot obtained from virus

infected plants and also confirmed by ELISA test.

The meristem cultured banana cv. Amritasagar plantlets were used as explant. Four levels of BAP (0, 3, 4 and 5 mg/l) and 5 levels of NAA (0.0, 1.0, 1.5, 2.0 and 2.5 mg/l) were used as treatment for shoot proliferation in experiment-I and four levels of IBA (0, 1, 2 and 3 mg/l) and 4 levels of NAA (0, 2, 3 and 4 mg/l) were used as treatment for root formation in experiment-II. ELISA test was conducted for the confirmation of virus eradication rate of regenerated plants and survival rate of regenerated plants in experiment-III.

ELISA Test

The ELISA test was conducted to confirm the virus free banana plants through meristem culture at the Microbiology Laboratory, Department of Plant Pathology, Bangabundho Sheikh Mujibur Rahman Agricultural University, Salna, Gazipur during

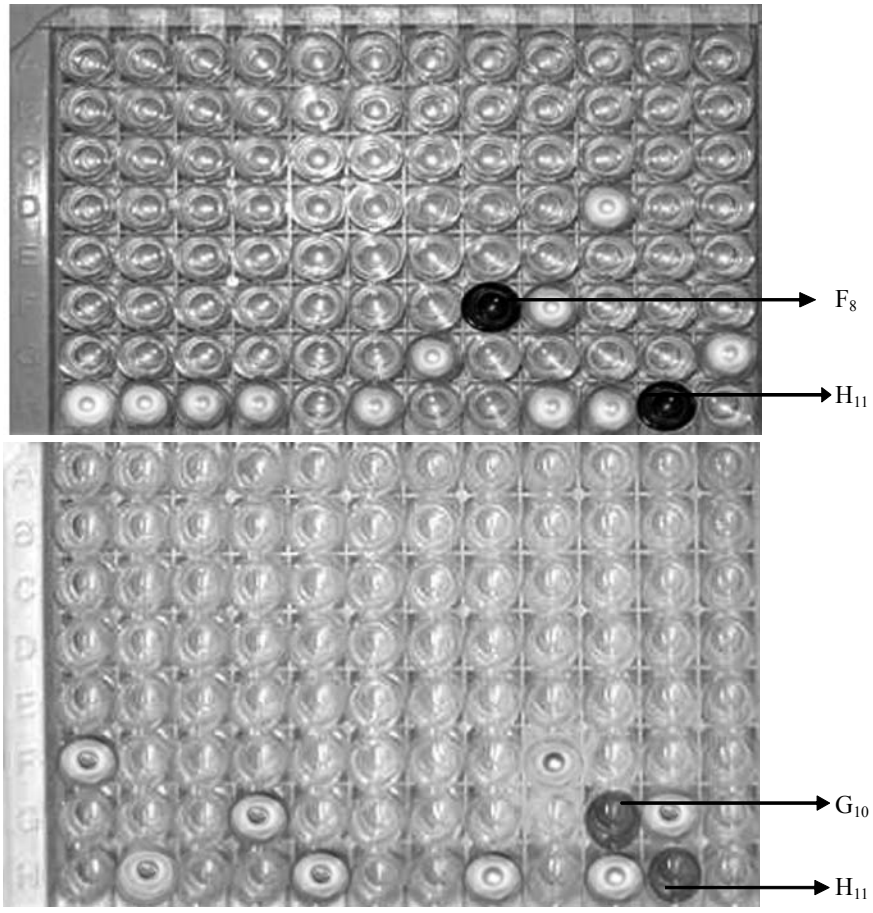

Plate 1. ELISA test for detection of virus in banana cv. Amritasagar

A) well of the micro titer plate after ELISA test (F_8 to H_{11} indicates BBTV sample)

B) well of micro titer plate after ELISA test (G_{10} to H_{11} indicates BMV sample).

March 16 to 23, 2004. The comparative results obtained in the investigation have been presented in Plate -1.

Steps in ELISA (Utilizing commercial kit: Agdia, USA)

Sample extraction buffer was prepared as per instruction supplied with the kit. Amount needed: 3000 ml (for 288 well or three 96 well micro titer plate) for safety allowance prepared about 500 ml. Disease sample (leaf, tissue etc.) and extraction buffer was mixed in 1:10 ratio eg. take 0.1 g tissue, extract with 1 ml extraction buffer in a mortar with pestle. Grinded it to make sap (discarding the debris) and dispensed in an append drop tube mark the tube. A loading diagram of the test was made (i.e. in what well which sample is to be placed) in a piece of paper.100 µl of the extracted sample in each well of the micro titer plate (pre coated with antibody) was dispensed. Also similar amount of solution was put for positive control and extraction buffer in two wells as check. The plate was incubated for 2 hrs in a humid box at room temperature or overnight at 4^{o}C. PBST (washing buffer) [Amount needed: 2 liter (for two time washing for 3 micro titer plate)], ECL buffer (conjugate buffer), enzyme-conjugate [Amount needed: 30-50 ml kit (to be prepared about just before beginning of washing the plates)] was prepared as per instruction supplied with the kit. The micro titer plate was washed with PBST (washing buffer): During washing the well was filled with buffer and quickly turned the plate upside down in whipping fashion on the sink and repeated the process several times (4 to 8) and finally dried on few sheets of paper towel or newsprint paper or blotting paper by repeated knocking. Dispensed 100 µl enzyme conjugate per well and the plate was incubated for 2 hrs in a humid box at room temperature. PNP (substrate for enzyme) buffer [Amount needed 30-50 ml] and PNP solution was prepared as per instruction supplied with the kit. The micro titer plate was washed with PBST again like previous system. 100 µl of PNP solution was dispensed per well and incubated in the humid box for 30-60 minutes.

Lay out and design of the experiment

The experiments were arranged in Completely Randomized Design (CRD) with 4 replications. Each of replications consisted of 5 culture vessels.

Number of shoots per explant

Number of shoots per explant was recorded at 10-15 days interval up to 40 days of culture and mean number of shoots per explant was calculated by using the following formula. $X = \frac{\Sigma \times i}{n}$ Where, X = Mean number of shoots/explant, $\times$i = Number of shoots/explant, n= Number of observations and Σ =Summation

Rate of Plantlets to Establishment

The percentage of plantlets established in the pot mixture of soil, sand, cowdung and coir were calculated using following formula:

Survival (%) = (Number of plantlets established in substrate / Total number of plantlets placed in substrate) × 100

Analysis of Data

The experiment was laid out in Completely Randomized Design (CRD) with four replications. The data recorded for different parameters were statistically analyzed using the "MSTAT" program. The differences between the treatment means were compared by the Least Significant Difference (LSD) test at 5% level of significance after performing ANOVA (GOMEZ and GOMEZ, 1983) for the interpretation of results.

Results and Discussion

Experiment-I: Effects of different concentrations and combinations of BAP and NAA on meristem cultured shoot proliferation and differentiation of banana cv. Amritasagar

To obtain plant regeneration and subsequent shoot proliferation from meristem cultured plantlets of banana cv. Amritasagar, MS medium supplemented with different concentrations and combinations of BAP and NAA were used in this experiment. Healthy and vigorous suckers were collected from the meristem cultured plantlets and cut into pieces having half stem and half rhizome. Moreover, a piece of 2.5-3.5 cm long and about 2 cm diameter plantlets were used as primary culture (explant) for shoot

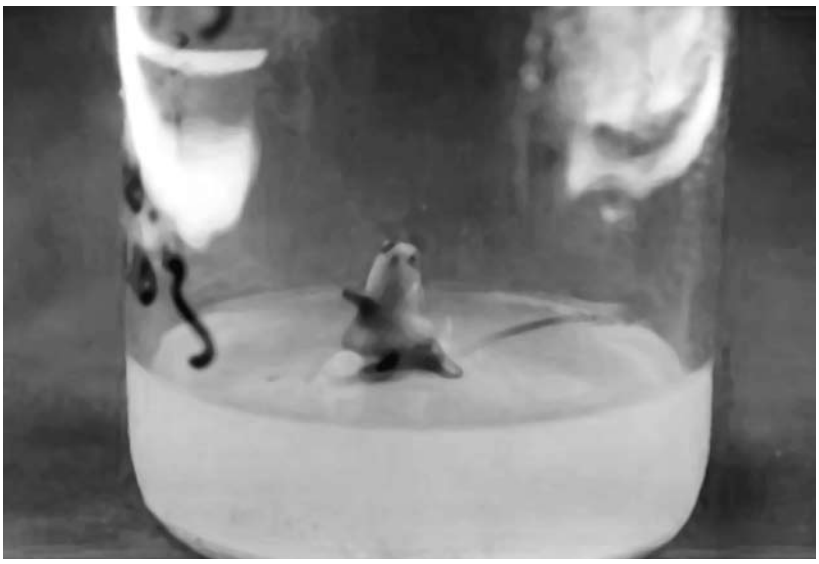

Plate2. Half shoot and rhizomatous explant for multiple shooting of banana cv. Amritasagar on MS media containing 4 mg/l BAP +2.5 mg/l NAA

proliferation (Plate 2).

Number of shoots per explant

The number of shoots produced per explant varied on MS media supplemented with different concentrations and combinations of BAP and NAA. The main effect of BAP on shoot proliferation was found significant. The results showed that 4 mg/l BAP gave the highest number of shoots at 15, 30 and 40 DAE. The highest number of shoots proliferated (3.76/explant at 40 DAE) with 4 mg/l BAP followed by 5 mg/l BAP (2.96/explant at 40 DAE) and the lowest number (1.52/explant at 40 DAE) was obtained from hormone free media (Fig. 1). The effect of NAA showed that the highest number of shoots (3.06/explant) obtained from MS medium supplemented with 1.5 mg/l NAA whereas, the lowest (2.20/explant) from MS medium without any NAA supplementation at 40 DAE. The results indicated that the increasing trend of shoot number was faster in 1.5 mg/l NAA than others (Fig 1).

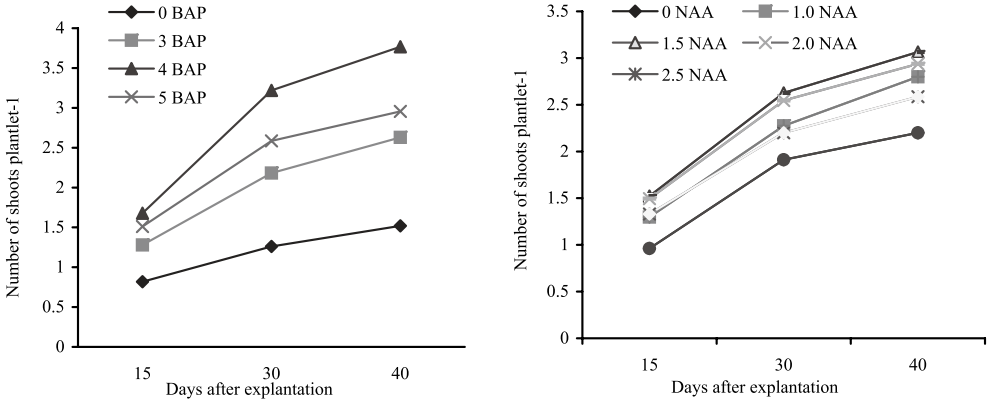

Fig. 1. Effect of different concentrations of BAP and NAA on shoot number of banana cv. Amritasagar at different days after explantation.

The combined effect of different concentrations and combinations of BAP and NAA on shoot proliferation has been presented in Table 1. Among the concentrations and combinations of MS media, BAP 4 mg/l + NAA 1.5 mg/l showed the highest shoot proliferation (4.58/explant) followed by BAP 5 mg/l treatment (3.30/explant) at 40 DAE (Plate 3). These results are in partial support of Rabbani et al. (1996) where they found that highest number of shoots per explants at 28 DAE was 3.11 ± 0.66 with 5 mg/l each of BAP and Kinetin. This variation might be due to the differences of species and inoculated explant. The explants cultured on MS medium without growth regulator did not produce any shoot. Similar results also reported by KHANOM *et al.* (1996) and REHANA (1999).

Table 1. Combined effect of different concentrations of BAP and NAA on shoot proliferation of banana plantlet cv. Amritasagar at different days after explantation

Treatment combination		Shoot number per plantlet at DAE		
BAP (mg/l)	NAA (mg/l)	15	30	40
0	0	0.00	0.00	0.00
	1.0	0.90	1.50	1.90
	1.5	1.14	1.65	1.98
	2.0	1.05	1.65	2.00
	2.5	1.00	1.50	1.71
3	0	1.00	2.10	2.55
	1.0	1.25	2.15	2.55
	1.5	1.40	2.25	2.75
	2.0	1.50	2.30	2.82
	2.5	1.25	2.10	2.48
4	0	1.30	2.55	2.95
	1.0	1.51	2.80	3.55
	1.5	2.10	4.00	4.58
	2.0	1.83	3.65	4.05
	2.5	1.65	3.10	3.70
5	0	1.55	3.00	3.30
	1.0	1.53	2.65	3.20
	1.5	1.45	2.60	2.95
	2.0	1.61	2.58	2.88
	2.5	1.42	2.10	2.45
LSD (0.05)		0.049	0.048	0.169
CV (%)		2.5	1.09	4.03

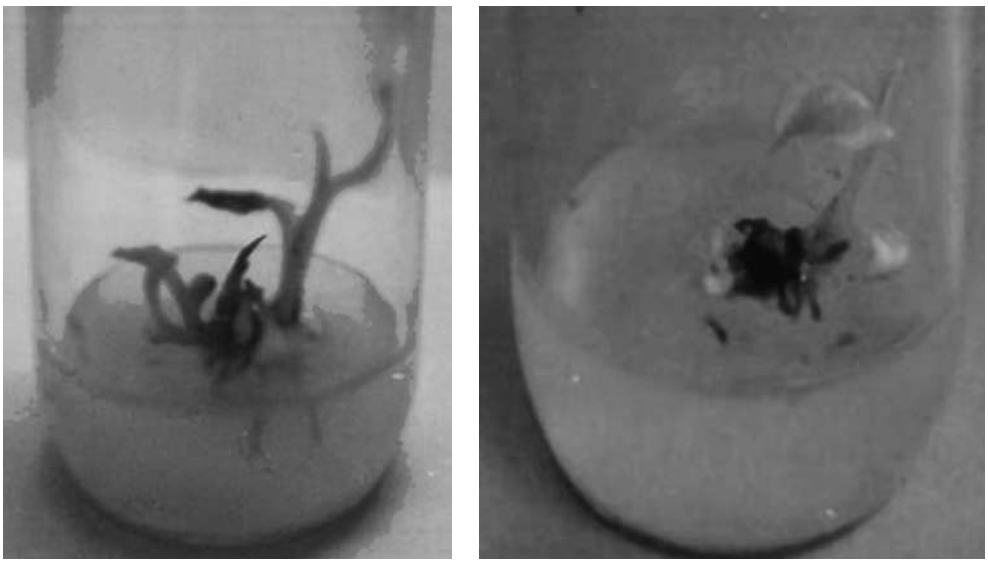

Plate 3. Multiple shoots of banana cv. Amritasagar produced on MS medium containing 4 mg/l BAP +1.5 mg/l NAA (left) and containing 2.5 mg/l NAA (right) at 40 days after explantation

Shoot length

The MS medium supplemented with BAP and NAA showed different results for increasing shoot length. Shoot length was significantly influenced by different BAP

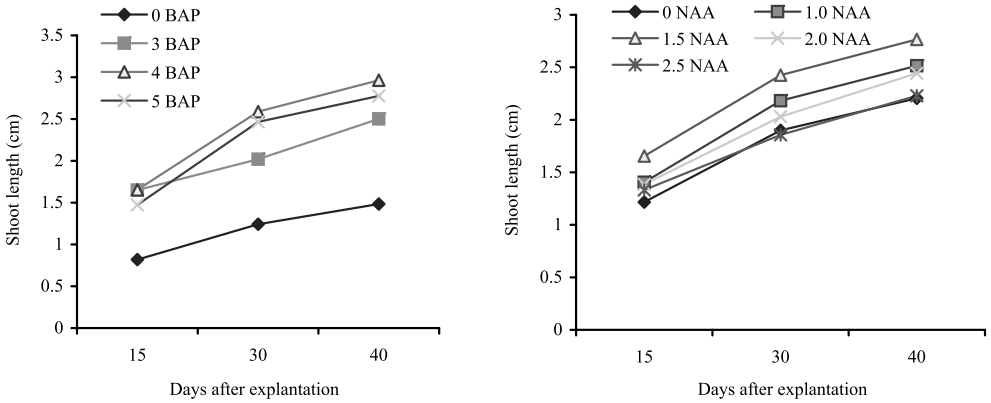

Fig. 2. Effect of different concentrations of BAP and NAA on shoot length of banana plantlet cv. Amritasagar at different days after explantation.

Table 2. Combined effect of different concentrations of BAP and NAA on mean shoot length of banana plantlet cv. Amritasagar at different days after explantation

Treatment combination		Mean shoot length (cm) at DAE		
BAP (mg/l)	NAA (mg/l)	15	30	40
0	0	0.00	0.00	0.00
	1.0	1.06	1.48	1.86
	1.5	1.22	1.65	1.98
	2.0	0.96	1.88	2.12
	2.5	0.85	1.20	1.46
3	0	1.25	1.75	2.25
	1.0	1.48	1.78	2.42
	1.5	1.72	2.12	2.41
	2.0	1.90	2.20	2.65
	2.5	1.90	2.25	2.78
4	0	1.90	2.92	3.22
	1.0	1.68	2.48	3.04
	1.5	2.03	2.98	3.56
	2.0	1.36	2.10	2.58
	2.5	1.30	2.10	2.42
5	0	1.72	2.93	3.35
	1.0	1.40	2.63	2.74
	1.5	1.65	2.95	3.12
	2.0	1.32	1.94	3.42
	2.5	1.28	1.88	2.25
LSD (0.05)		0.049	0.138	0.048
CV (%)		2.22	4.34	1.03

levels at 15, 30 and 40 DAE. At 15 DAE, the longest shoot (1.65 cm) was obtained from 4 mg/l BAP followed by 3 mg/l BAP (1.65 cm) supplemented MS medium. Finally, at 40 DAE the longest shoot (2.96 cm) was found from 4 mg/l BAP that was identical with the shoot length obtained from 5 mg/l BAP (2.77 cm) supplemented MS medium (Fig. 2). At 15, 30 and 40 DAE, similar result was found in case of NAA where highest shoot length (2.77 cm at 40 DAE) was obtained from 1.5 mg/l NAA supplemented MS media. Moreover, fluctuated results were observed when the media was supplemented with 1.0, 2.0 and 2.5 mg/l NAA (Fig. 2). The smallest shoot (2.21 cm) was recorded in the medium supplemented without NAA.

The combined effect of different concentration of BAP and NAA on the increment of shoot length of banana cv. Amritasagar have been presented in Table 2. Various concentrations and combinations of supplements showed significant variation in shoot length of banana plantlets. Among the combinations used, 4 mg/l BAP + 1.5 mg/l NAA showed the longest shoot (3.56 cm) followed by 5 mg/l BAP (3.35 cm) treatment. Rabbani *et al.* (1996) found similar results from 5.0 mg/l BAP supplemented MS media. During the culture period, it was also noted that there was fluctuation in increment of shoot length with all the media supplemented with different concentrations of BAP and NAA alone or in combination. Therefore, the rate of increase was faster in 4 mg/l BAP + 1.5 mg/l NAA supplemented media (Table 2). The results of present experiment agreed with the findings of KHANAM *et al*. (1996) who obtained the longest shoot in banana on MS medium supplemented with 25 µM/l BAP treatments.

Leaf Number per plantlet

The number of leaves produced per plantlet was also influenced by the different concentrations and combinations of BAP and NAA. The effect of BAP showed significant variation in leaf number of banana plantlets. The maximum number of leaves (2.78/ plantlet) counted from the medium supplemented with 3 mg/l BAP which was identical with that of explant cultured on the MS medium containing 5 mg/l BAP at 40 DAE (Fig.

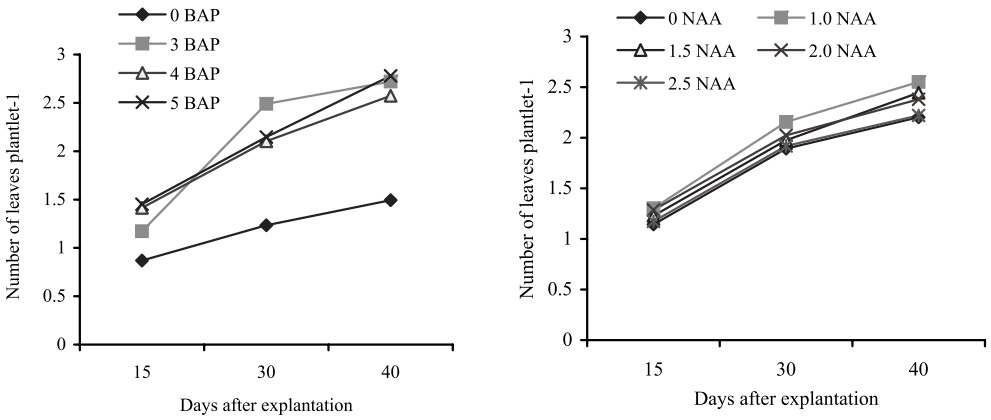

Fig. 3. Effect of different concentration of BAP and NAA on number of leaves of banana plantlet cv. Amritasagar at different days after explantation.

3). At 40 DAE significant influence was found with different concentrations of NAA. The highest number of leaves (2.55/plantlet) obtained from 1.5 mg/l NAA, whereas the lowest (2.20/plantlet) without NAA.

The combined effect of different concentrations of BAP and NAA has been presented in Table 3. The results showed that the maximum number of leaves (3.11/ plantlet) produced with 4 mg/l BAP+1.5mg/l NAA, which was identical with the treatment of 5 mg/l BAP. The results of the present experiment also agreed with the findings of Rabbani et al. (1996) who obtained the same results from 5.0 mg/l BAP. The lowest number of leaves (1.78/plantlet) obtained from 1.0 mg/l NAA supplemented MS media (Table 3).

Longest leaf length

A significant variation in leaf length was noted among the different concentrations and combinations of BAP and NAA at different DAE. It was observed that the media containing 3 mg/l BAP produced the longest leaves (2.12 cm), which was identical with the explant cultured on the medium containing 4 mg/l BAP (2.10 cm) at 40 DAE. During the culture period, there was a sharp increase trends in leaf length for the main

Table 3. Combined effect of different concentrations of BAP and NAA on mean leaf number banana plantlet cv. Amritasagar at different days after explantation

Treatment combination		Mean leaf number per plantlet at DAE		
BAP (mg/l)	NAA (mg/l)	15	30	40
0	0	0.00	0.00	0.00
	1.0	1.15	1.48	1.78
	1.5	1.10	1.48	1.96
	2.0	1.09	1.62	1.89
	2.5	1.00	1.58	1.84
3	0	1.24	2.49	2.83
	1.0	1.08	2.44	2.75
	1.5	1.12	2.38	2.57
	2.0	1.22	2.60	2.92
	2.5	1.20	2.53	2.84
4	0	1.63	2.40	2.88
	1.0	1.40	1.98	2.49
	1.5	1.60	2.47	3.12
	2.0	1.36	1.88	2.38
	2.5	1.27	1.78	2.0
5	0	1.70	2.68	3.10
	1.0	1.52	2.18	2.80
	1.5	1.39	2.29	2.56
	2.0	1.24	1.80	2.33
	2.5	1.22	1.78	2.20
LSD (0.05)		0.049	0.109	0.154
CV (%)		2.13	3.52	4.18

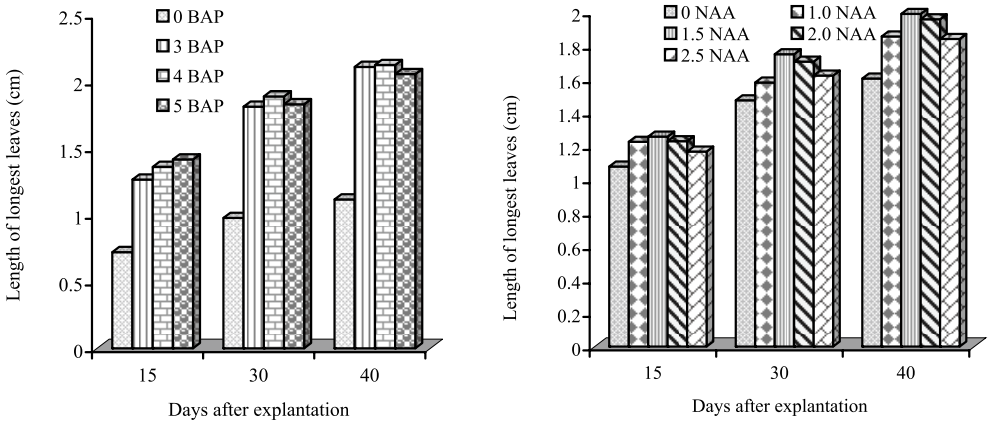

Fig. 4. Effect of different concentrations of BAP and NAA on length of longest leaves of banana cv. Amritasagar at different days after explantation.

Table 4. Combined effect of different concentrations of BAP and NAA on mean length of longest leaf of banana plantlet cv. Amritasagar at different days after explantation

Treatment combination		Mean length of longest leaf (cm) at DAE		
BAP (mg/l)	NAA (mg/l)	15	30	40
0	0	0.00	0.00	0.00
	1.0	0.99	1.23	1.38
	1.5	0.89	1.16	1.41
	2.0	0.82	1.15	1.33
	2.5	0.91	1.35	1.46
3	0	1.23	1.78	2.11
	1.0	1.18	1.62	1.91
	1.5	1.22	1.87	2.15
	2.0	1.41	1.98	2.35
	2.5	1.28	1.81	2.10
4	0	1.48	1.98	2.09
	1.0	1.35	1.81	2.09
	1.5	1.52	2.12	2.29
	2.0	1.29	1.89	2.18
	2.5	1.18	1.65	1.90
5	0	1.60	2.14	2.22
	1.0	1.38	1.67	2.05
	1.5	1.40	1.86	2.12
	2.0	1.40	1.81	1.98
	2.5	1.31	1.68	1.91
LSD (0.05)		0.049	0.048	0.048
CV (%)		1.96	1.39	1.33

effect of BAP and NAA (Fig. 4). Highest leaf length (1.99 cm) was obtained from 1.5 mg/l NAA supplemented MS media and smallest length (1.61cm) was recorded in the media supplemented without NAA at 40 DAE.

The combined effect of BAP and NAA on leaf length on banana plantlets has been presented in Table 4. Among the combinations, BAP 3 mg/l + NAA 2.0 mg/l containing MS media produced the longest leaf (2.35 cm) followed by 4 mg/l BAP+1.5 mg/l NAA (2.29 cm) at 40 DAE. Therefore, the lowest leaf length (1.33 cm at 40 DAE) was recorded from the treatment of 2.0 mg/l NAA. REHANA (1999) also reported the similar result.

Number of roots per explant

The effect of BAP showed that there was negative relationship between rooting and BAP application, the highest number (2.82/explant) of roots produced on the medium without BAP supplementation, which was identical with the treatment of 5 mg/l BAP at 40 DAE. Therefore, the number of roots was decreased with the increase of BAP concentration at different DAE (Fig. 5). The effect of NAA was found significant at different DAE. The highest number of roots (2.78/explant at 40 DAE) was obtained by 2.0 mg/l NAA. The lowest number of roots (1.92/explant) was recorded with 1.0 mg/l NAA, where no response was taken in control treatment. The results showed that the number of roots gradually increased with the increase of NAA concentrations at different DAE (Fig. 5).

The combined effect of BAP and NAA on root number, a small number of roots was produced by different concentrations at 15 DAE. Moreover, control with the other treatments showed absence of root at 15 DAE. At 30 and 40 DAE similar result was found, where the highest root number (4.19/explant) was obtained with 2.0 mg/l NAA at 40 DAE, which was significantly higher over other treatments (Plate 4 and Table

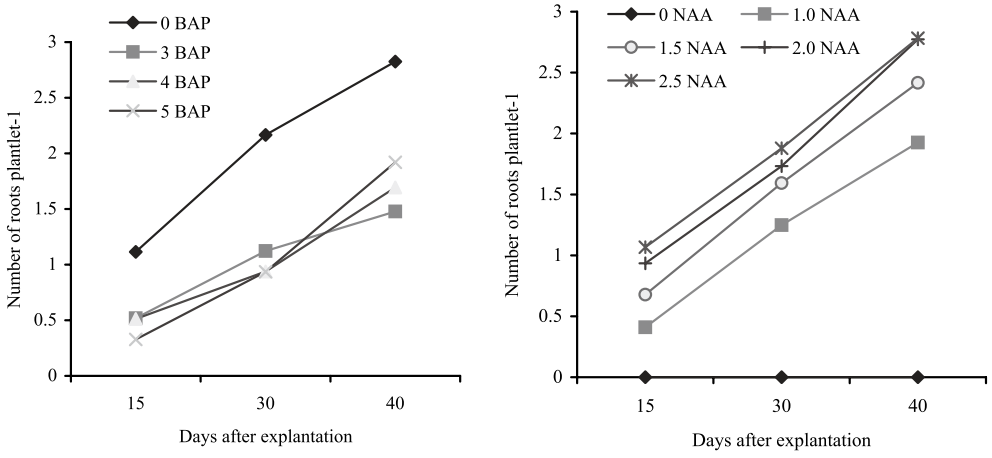

Fig. 5. Effect of different concentrations of BAP and NAA on number of roots of banana plantlet cv. Amritasagar at different days after explantation. Control treatment (without NAA) did not show any root formation.

Table 5. Combined effect of different concentrations of BAP and NAA on mean root number of banana plantlet cv. Amritasagar at different days after explantation

Treatment combination		Mean root number per plantlet at DAE		
BAP (mg/l)	NAA (mg/l)	15	30	40
0	0	0.00	0.00	0.00
	1.0	1.26	2.35	2.95
	1.5	1.31	2.75	3.33
	2.0	1.50	2.90	4.19
	2.5	1.50	2.83	3.65
3	0	0.00	0.00	0.00
	1.0	0.00	0.83	1.32
	1.5	0.51	1.21	1.88
	2.0	0.98	1.67	2.11
	2.5	1.10	1.90	2.08
4	0	0.00	0.00	0.00
	1.0	0.38	0.91	1.44
	1.5	0.58	1.33	2.05
	2.0	0.65	1.08	2.32
	2.5	0.95	1.37	2.65
5	0	0.00	0.00	0.00
	1.0	0.00	0.90	1.99
	1.5	0.31	1.08	2.40
	2.0	0.61	1.28	2.51
	2.5	0.71	1.49	2.71
LSD (0.05)		0.049	0.048	0.049
CV (%)		5.89	2.86	1.31

5). The lowest root number (1.32/ plantlet) was gained with 3 mg/l BAP +1.0mg/l NAA. The present results indicated that treatment 2.0 mg/l NAA was suitable for root formation, which is similar with the findings of GUBBUK and PEKMEZCI (2001).

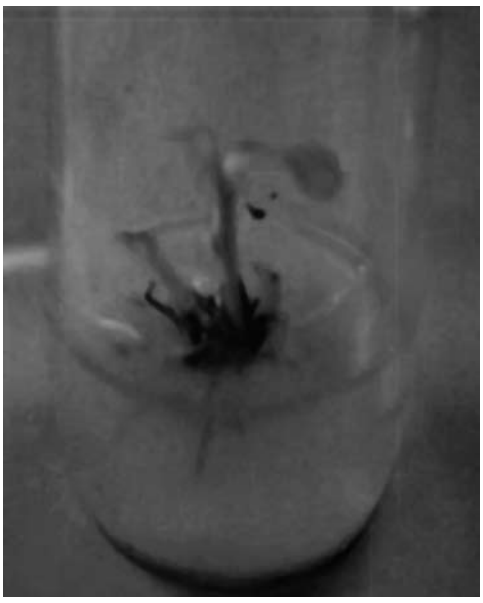

Plate 4. Root formation of banana plantlets cv. Amritasagar on MS medium containing 0 mg/l BAP+2.0 mg/l NAA at 40 days after explantation.

Experiment-II: Effects of different concentrations and combinations of IBA and NAA on rooting of meristem cultured banana plantlet cv. Amritasagar

The meristem cultured shoots were collected from *in vitro* grown plants (used in expt.-I). When those were subcultured on half strength of MS medium supplemented with different levels of IBA (0, 1, 2 and 3 mg/l) and NAA (0, 2, 3 and 4 mg/l) in order to allow root formation. Some roots were developed from the proliferated shoots. Root numbers varied with different concentrations and combinations of IBA and NAA.

Number of roots

The prolific shoot of banana cv. Amritasagar was cultured on half strength of MS media supplemented with different levels of IBA (0, 1, 2 and 3 mg/l) and NAA (0, 2, 3 and 4 mg/l). The effect of IBA was found significant at different DAE. At 30 DAE, the highest number of roots (5.10/explant) of banana cv. Amritasagar was found from 2 mg/l IBA, followed by 1 mg/l IBA. The lowest number of roots (1.13/explant) at 30 DAE was produced by MS medium without IBA supplementation. Moreover, at 10 and 20 DAE, the root number was increased with the increase of IBA concentration up to 2 mg/l (Fig. 6). The effect of NAA showed significant result at 10, 20 and 30 DAE, whereas 3 mg/l NAA represented highest number of roots (5.66 /explant) at 30 DAE and lowest number of roots (2.75/explant) at 30 DAE was produced by MS media without NAA supplementation. So the present result reveals that NAA is superior to IBA in respect to root formation.

At different DAE, the combined effect of IBA and NAA also showed significant variation in root number. The highest number of roots (6.98/explant) was obtained from 2 mg/l IBA + 2 mg/l NAA and the lowest (1.65/explant) from control at 30 DAE (Table 6 and Plate 5). Domingues *et al*., (1995) reported that rooting was stimulated in a nutrient solution containing 0.1 mg/l NAA or IBA. The result of the present study was in consisted with the findings of Khanam *et al*. (1996).

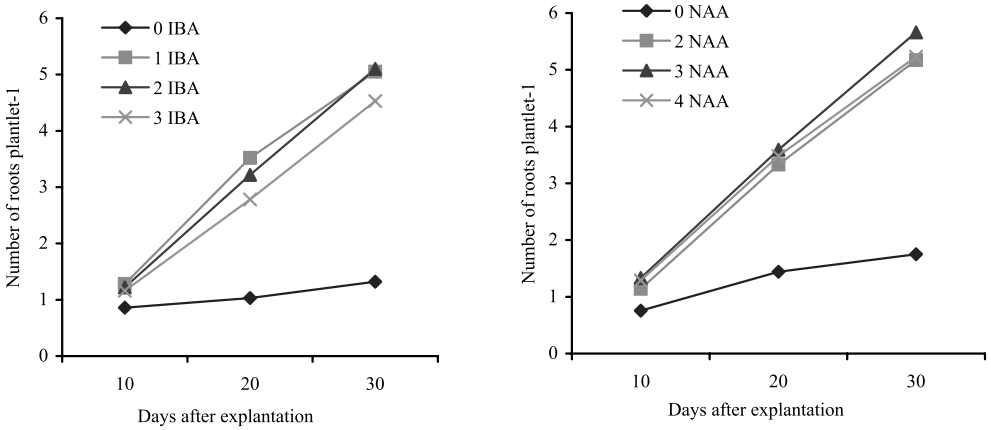

Fig. 6. Effect of different concentrations of IBA and NAA on number of roots of banana cv. Amritasagar at different days after explantation.

Table 6. Combined effect of different concentrations of IBA and NAA on root number of proliferated shoot of banana cv. Amritasagar at different days after explantation

Treatment combination		Root number per plantlet at DAE		
BAP (mg/l)	NAA (mg/l)	10	20	30
0	0	0.45	0.98	1.65
	2	0.90	2.93	4.68
	3	0.99	3.09	4.85
	4	1.10	3.12	5.35
3	0	0.77	1.60	2.90
	2	1.20	3.25	5.05
	3	1.49	4.50	6.60
	4	1.65	4.72	5.65
4	0	0.92	1.98	3.05
	2	1.35	4.26	6.98
	3	1.40	3.37	5.37
	4	1.21	3.29	5.01
5	0	0.88	2.01	3.42
	2	1.10	2.91	3.99
	3	1.45	3.41	5.81
	4	1.20	2.81	4.92
LSD (0.05)		0.051	0.049	0.050
CV (%)		2.52	0.91	0.43

Plate 5. In vitro Plantlet with vigorous Roots of banana cv. Amritasagar grown on MS medium supplemented with 2 mg/l IBA + 2 mg/l NAA at 30 days after explantation.

Root length

A significant variation in the root length developed by the plantlets was influenced considerably by different concentrations and combinations of IBA and NAA (Table 7). The effect of IBA showed significant variation in root length at different DAE. The longest roots (4.18 cm) were observed with 3 mg/l IBA followed by 2 mg/l IBA, whereas the shortest (1.87 cm) was obtained from MS medium without IBA at 30 DAE. On the contrary, the longest root (4.23 cm) was observed with 3 mg/l of NAA

Table 7. Combined effect of different concentrations of IBA and NAA on root length and vigour of regenerated root of proliferated shoot of banana cv. Amritasagar at different days after explantation

Treatment combination		Root length (cm) at DAE			Vigour of regenerated root
BAP (mg/l)	NAA (mg/l)	10	20	30	
0	0	0.30	1.00	1.95	+
	2	0.50	1.98	2.55	+
	3	1.03	2.25	4.01	+++
	4	0.71	1.49	2.99	++
3	0	0.38	1.28	1.60	+
	2	0.65	1.90	3.11	++
	3	1.05	2.23	3.90	+++
	4	0.68	1.80	2.91	++
4	0	0.75	2.65	3.80	++
	2	0.77	2.09	3.29	++
	3	1.01	2.98	4.01	+++
	4	0.83	2.55	3.83	+++
5	0	0.66	2.95	3.80	++
	2	0.87	2.72	4.02	+++
	3	1.11	3.45	5.00	+++
	4	0.92	2.41	3.89	++
LSD (0.05)		0.050	0.160	0.016	-
CV (%)		2.08	0.73	0.54	-

Legend: + indicate light green,
++ indicate green and
+++ indicate dark green

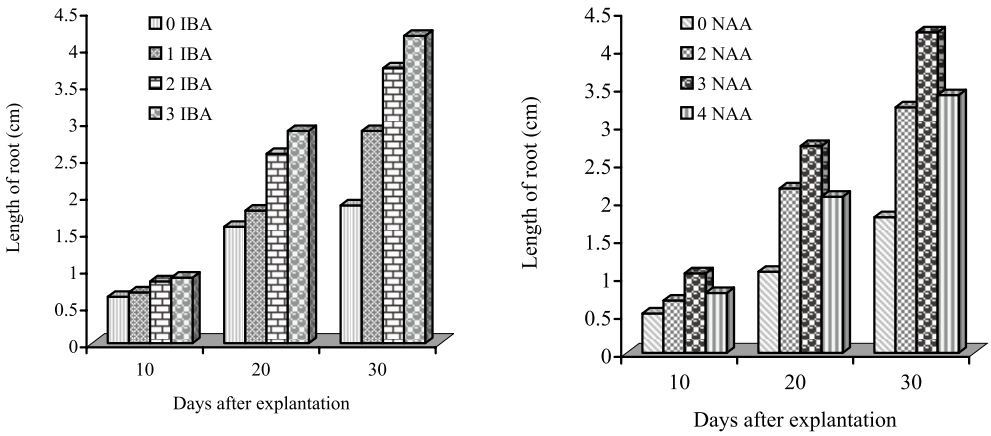

Fig. 7. Effect of different concentration of IBA and NAA on length of root of banana cv. Amritasagar at different days after explantation.

and the shortest (1.78 cm) was obtained from MS medium without NAA at 30 DAE. The result indicated that there was a sharp increasing trend in root length at different

DAE (10, 20 and 30 DAE). Moreover, root length increased with the increase of IBA and NAA concentration up to 2 and 3 mg/l, but the increasing rate was considerably faster in case of 2 and 3 mg/l IBA and NAA respectively (Fig. 7).

The combined effect of IBA and NAA also showed significant variation on root length at different DAE. The length of longest root (5 cm) was obtained from 3 mg/l IBA +3 mg/l NAA and the shortest length of roots (1.6 cm) from 1 mg/l IBA in combination with control at 30 DAE (Table 7 and plate 6). The present result partially agreed with the findings of Gubbuk and Pekmezci (2001) who reported that 1.0 µM/l IBA in MS medium was sufficient for rooting. The results are similar with the findings of KHANAM *et al*. (1996) who found longest roots (4.2 cm) of banana by adding 2.0 mg/l IBA in the media.

Plate 6. Rooted plantlets of banana cv. Amritasagar on MS media containing 2 mg/l IBA (left) and 3 mg/l IBA + 3 mg/l NAA (right) at 30 days after explantation.

Root vigour

The root vigour was recorded by eye estimation. It was influenced by application of different levels of IBA and NAA (Table 7). Root initiated from the 2 and 3 mg/l concentrations of IBA were more vigorous compared with other treatments. It was also observed that more vigorous roots produced from 2, 3, and 4 mg/l NAA treatment compared to without NAA (Plate 6). The present result agreed with the findings of HABIB (1994), who also obtained more vigorous roots with 2.0 mg/l IBA in variety Sabri, whereas, ALI (1996) was from banana cv. Amritasagar.

Experiment –III (a): Eradication rate of virus free banana plants cv. Amritasagar regenerated from meristem cultured by using ELISA test

MOREL *et al.* (1952) first obtained virus free Dahlias and Potatoes with the help of meristem culture. Initially, the health status of explants and regenerated plants were checked through ELISA. Collected explants were fully virus infected which was confirmed by ELISA test, because the wells of micro titer plate showed colour, indicate positive results i.e. explants virus infected. The wells were examined by eye or measured on a micro titer reader at 405 nm. On the contrary, wells of micro titer plate of regenerated plants after ELIS test in which colour developed, indicate positive result i.e. regenerated plants virus infected and in which wells no colour developed, indicate negative results i.e. regenerated plants was not virus infected or virus free plate-1. Test result was valid on the basis of positive control well gave a positive colour and buffer well remained clear. In this experiment, no colour development result was significant because regenerated plants were virus free. The regeneration rate of banana bunchy top virus (BBTV) free plants were 57.14 % and for banana mosaic virus (BMV) free plants were 64.28% (Table 8). The present results agreed with the findings of HELLIOT B. *et al*. (2002) who also reported on successfully BMV eradication by cryopreservation of highly proliferated meristem of banana (cv. Williams BSJ, ITC 0570, AAA). The Thirty percent of the regenerated plants were found to be healthy by DAS- ELISA. BMV eradication rate was also achieved by GUPTA (1986) in approximately 100% of the regenerated plants when using meristem cultured in combination with a two week heat therapy (38- 40ºc). Similar results also reported by BONDOK *et al*. (1987). RAMOS *et al*. (1990) reported that elimation rate of banana bunchy top virus (*Musa* sp. cv. Lacatan) by heat pre-treatment (40ºc for 16 hrs. photoperiod and 32ºc for 8 hrs. darkness) with meristem culture, result yielded 59.3% and 68.2% BBTV free plants, respectively. Results from other studies also showed that regenerated banana plants by meristem culture eradicated BBTV and BMV to great extent (MORI *et al*.1977, BHOJWANI *et al*. 1983, LOGON *et al*.1985, ALLAM *et al.* 2000).

Table 8. Eradication rate of virus free banana cv. Amritasagar by meristem cultured after ELISA test

Variety	Name of diseases	Number of plants tested	Number of virus free plants	Number of virus infected plants	% of virus free plants
Amritasagar	Banana bunchy top virus(BBTV)	14	8	6	57.14
	Banana mosaic virus (BMV)	14	9	5	64.28

Experiment-III (b): Survival rate of regenerated ex vitro hardened plants from meristem cultured of banana cv. Amritasagar in different pot mixtures

After rooting, the lids of the culture vessels were opened slightly and kept in growth room for five days and then transferred to laboratory at room temperature for another two days for hardening. After that the plantlets with well developed roots were

Plate 7. Hardened in vitro grown plantlets of banana cv. Amritasagar (above row) and Sabri (below row) after 15 days of transfer to polyethylene bag.

Plate 8. Virus free healthy plant of banana cv. Amritasagar established in pot mixture (sand : soil : cow dung = 1:1:1 and coir : soil = 1: 2) after 40 days of transfer to earthen pot.

Table 9. Survival rate of regenerated plant from meristem cultured of banana cv. Amritasagar transferred to the pot with different soil mixture

Variety	Number of plantlets potted	Number of plants survived	Pot mixture	% of survival plants
Amritasagar	6	5	Sand : Soil : Cowdung = 1:1:1	83.33
	6	4	Coir: Soil = 1:2	66.67

removed from the culture vessels without damaging the roots. The agar was washed away from the roots with running tap water. After that, the plantlets were treated with fungicide (Ridomil) for few seconds and transferred to small polyethylene bag filled with soil, sand and well decomposed cow dung (1:1:1) and coir & soil (1:2) as well as kept in the hardening room for 3-4 days (Plate 7). Then the polyethylene bag containing plantlets were transferred to net house, where proper care was taken for growth and development of banana plantlets. After 10-15 days, maximum plants showed vigorous growth while some were less vigorous (Plate 7). Therefore, the plantlets became well hardened and ready to replace in the field conditions after 30 days (Plate 8). The survival rate of plantlets was about 83.33% in sand, soil and cow dung (1:1:1) pot mixture and 66.67% in coir and soil (1:2) pot mixture (Table 9). The findings are in agreement with the result of Dilip *et al.* (2002) who reported that more than 25,000 plantlets produced through tissue culture were transferred to the field with survival rate 90%. The well developed plantlets were hardened for the establishment in the field by Jasari *et al.* (1999) who showed that average 92% of plantlets survived. Azad and Amin (1999) also mentioned that survival rate of the plantlets under ex vitro condition was 80% in pot mixture containing sand, soil and compost at 1:1:1 ratio. Similar results also reported by (Cronauer and Krikorian 1984, Gupta 1986).

Based on the findings of this study it may be concluded that for rapid shoot proliferation, the highest number of shoots, the longest shoots and the highest number of leaves were recorded in the media containing 4 mg/l BAP + 1.5 mg/l NAA. On the contrary, plantlets were cultured on half strength MS media supplement with different levels of IBA and NAA. For rapid root formation, and the highest number of more vigorous roots were obtained from 2 mg/l IBA +2 mg/l NAA and the treatment 3 mg/l IBA+3 mg/l NAA produced the longest roots at 30 DAE. The effect of BAP on rooting was negative. The numbers of roots were decreased with the increase of BAP concentrations. On the other hands, the number of roots gradually increased with the increase of NAA concentrations but the effect of IBA was found significant at different DAE. Shoot excised meristem of regenerated plants of banana cv. Amritasagar were tested either they were virus free or not by ELISA test. In vitro meristem culture produced 57.14% BBTV free plants and 64.28% BMV free plants. The survival rate of regenerated plants were found to be better 83.33% in a pot mixture of sand, soil and cowdung (1:1:1) compared to 66.67% in pot mixture of coir and

soil (1:2). In Bangladesh, very few investigations have yet been carried out on this study. The present study was indicated that to find out the suitable concentrations and combinations of appropriate PGRs for the regenerations of virus free healthy planting materials of banana cv. Amritasagar for use of farmer's level through in vitro meristem culture that may be helpful for commercial exploitation.

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