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IN-VITRO PROPAGATION AND ELISA BASED SCREENING FOR THE DEVELOPMENT OF BANANA BUNCHY TOP VIRUS FREE PLANTS

Hira Yasin[1], Muhammad Aslam Javed[2], Shahid Nazir[2], Rohina Bashir[1] and Muhammad Arif Khan[2]
[1]Department of Botany, Government College University Faisalabad, Faisalabad, Pakistan
[2]Agricultural Biotechnology Research Institute, Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan
E-Mail: shahidnazir97@yahoo.com

ABSTRACT

Banana (*Musa spp*.) is a chief source of carbohydrates and minerals that cultivated in various parts of the world including Pakistan. This important food crop is affected by a number of pathogens including viruses' especially banana bunchy top virus which causes bunchy top disease and responsible for major loss of yield annually. The production of disease free banana plants is therefore the pre-requisite for the promotion of this crop in Pakistan. A research work was planned to develop disease free plants through in-vitro micro-propagation using shoot tip explant. Variety of hormones at various levels were practiced to enhance the multiplication rate of banana cv Basrai. MS medium supplemented with 7.5 mg L^{-1} BAP and 1.0 mg L^{-1} NAA was found to be the most suitable for shoot multiplication and 0.5 mg L^{-1} of IAA best for the initiation of roots. Shoot tip derived plantlets then transferred to pots containing 1:1 of (sand and clay) for hardening and acclimatization in greenhouse and established plantlets were exposed to natural environment in pots. The leaf samples of in-vitro developed plants were screened against bunchy top virus through ELISA and found that 90% plantlets were virus free.

Keywords: banana bunchy top virus, tissue culture, shoot tip, banana, enzyme linked Immunosorbent assay.

INTRODUCTION

Banana is an important foodstuff of the world and is grown over an area of around five million hectare with an annual production of more than 70 million tons [6]. Banana and plantain (Musa spp.) belonging to the family Musaceae, are one of the world's most significant survival crops and grown in 130 countries which is more than any other fruit crop with production of around 100 million tons per year. It is a rich source of carbohydrates, fiber and protein with minimum percentage of fat. Pakistan stood at 6th position for the area and production of banana crop. In year 2010-11 it was cultivated on an area about 29.6 thousands hectare with production of 141.2 thousand tons (Pakistan Bureau of Statistics, 2010-11). The single cultivar which is Cavendish dwarf commonly called Basrai is cultivated in Pakistan and covers nearly the 90% area of banana crop. Average production of bananas in Asian countries is 18.3 tons per hectare, while it is just 4.3 tons in Pakistan (Published in the Express Tribune, February 10th, 2011). Usually, it is propagated through suckers which produce as off shoots with the mother plant. This crop meets consistent challenges of large numbers of natural and unnatural afflicts [9]. Among natural afflicts, Banana bract mosaic disease caused by Banana bract mosaic virus, Banana mild mosaic disease is Banana mild mosaic virus, Banana bunchy top virus (BBTV) cause the Banana Bunchy Top Disease (BBTD) are severely affecting banana production worldwide [1, 16]. Among these BBTD is one of the most important diseases of banana cause a huge loss over the world annually. This disease is transmitted through suckers, so new fields could also be contaminated with this virus. Furthermore, *Pentalonia nigronervosa* (black aphid) a carrier/vector, is also present in the fields causing additional dispersion of BBTV. Heliconia, Strelitzia and Ravenala are alternate hosts of these aphids while they mainly form colonies on banana [9]. There are many areas including Asia, Africa and Pacific region facing the BBTD, while this is not found in Central and South America and the Caribbean [5]. The first report on BBTD was published in 1889 in Fiji and massive damage affects their industry [7].

A range of techniques/methods are being used for the development of BBTD free banana plants. Among these in-vitro shoot-tip culture is a central methodology currently used for the production of disease free banana plants and propagation of newly bred clone [12]. Assani *et al*. (2003) investigated successful formation of haploid plants of *Musa balbisiana* (BB), which is familiar for the inhibition against economically important diseases of banana because they have genes specific for disease tolerance. Protoplast fusion techniques become useful in introducing the varieties which inhibit the disease from wild to cultivated varieties.

The earliest investigations about the micropropagation of banana were made by Taiwan in the 70's [8]. Micropropagation of banana can be done by shoot tip [4] and with apices of male floral part. It has been observed that the rate of multiplication of banana depends upon the genotype as well as in vitro behavioral difference [10]. Banana taken from this method are stronger, productive and bearing good quality fruits than those which are taken from the other methods [11]. Keeping in view, the present research work was planned to develop disease free banana plants through in-vitro techniques using shoot tip as an explant. Various hormones were used in different combinations and concentrations to investigate the multiplication rate of banana cv. Basrai.

MATERIALS AND METHODS

Explants preparation and sterilization

The explants developing shoot tip, was taken from the suckers of Cavendish dwarf (Basrai). Soil was removed by washing the suckers with distilled water for 15 minutes. Roots and outer tissues were detached with sterilized surgical blade. The suckers were peeled off upto the size of 5x5cm in length containing one shoot tip. Each explant was surface sterilized with 50 % Sodium hypochlorite, and few drops of tween-20 for 25 minutes. Following sterilization, explants were washed thrice with sterilized distilled water and aseptically cleaned and cut into 5x5mm size of shoot tip.

In-vitro multiplication media

The MS medium supplemented with 4.33 mg L^{-1} of MS salts [13], 30g L^{-1} sucrose and (1.75 mg L^{-1}) of phytagel was used. The pH of the medium was adjusted to 5.7 prior to autoclaving. The medium were poured into glass test tubes and autoclaved at 15 lbs for 20 minutes at 121^{o}C.

For multiplication of plantlets various hormones including BAP, NAA, IAA and IBA with different concentrations and combinations were used in MS medium. Effect of hormones on shoot multiplication of cv. Basrai was assessed by using different levels of both BAP and NAA starting from 0, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 10.0 mg L^{-1} of BAP and 0, 0.5, 1.0, 1.5 and 2.0 mg L^{-1} of NAA separately and in combinations. Root initiation was checked at two levels of IAA (0 and 0.5 mg L^{-1}) and five levels of IBA (0, 0.5, 1.0, 1.5 and 2.0 mg L^{-1}). The sterilized shoot tip was then aseptically placed in culture tubes and incubated at 25^{o}C with 16 hours light and 8 hours dark cycle and light intensity was maintained at 2000 lux.

In-vitro sub-culturing of plantlets

After four weeks when the plantlets grown enough to subculture, the number of leave and shoot length were measured. Five subcultures were done and at each subculture number of leaves, number of shoots and shoot length and number of roots were measured after 10 days interval.

Statistical analysis

The data collected on various parameters were subjected to analysis of variance [15]. The difference between treatments and their interaction with significant mean values was compared by Duncan's Multiple Range test.

Acclimatization of in-vitro raised banana plants

In-vitro developed plantlets were acclimatized in pots using a mixture of soil and sand in ratio of 1:1. Each pot then covered with the polythene bag in order to control the humidity. After one week the bags were removed and plantlets were exposed to natural environments in filed.

Screening of BBTV free plantlets using ELISA

Enzymes Linked ImmunoSorbent Assay (ELISA) was performed to screen the virus free banana plants. The acclimatized plants were analyzed for the presence of banana bunchy top disease using plate test and disease free plants were screened.

RESULTS

Effect of BAP and NAA on number of leaves

After the initiation of culture, the explants were transferred to the media supplemented with different concentrations of BAP and NAA. Data was recorded from 30 in-vitro grown plants for each traits and average mean were measured. The results showed that BAP has significant effect on the number of leaves as compared to NAA and the interaction between BAP and NAA is also significant but the number of leaves varies under different concentrations of BAP and NAA (Table-1). Maximum number of leaves (5.67 leaves/explants produced at 10 days) was obtained using 7.5 mg L^{-1} BAP and 1 mg L^{-1} of NAA. After 20 days 7.39 leaves/explants were generated using same concentrations of hormones.

Effect of BAP and NAA on shoot multiplication

The response of shoot multiplication was different in different concentrations of BAP and NAA. Results showed that highest number of shoots produced in MS medium supported with 7.5 mg L^{-1} BAP and 1 mg L^{-1} NAA 6.47, 8.33 and 13.0 shoots/explants after 10, 20 and 30 days of culture respectively (Table-1). The second greater number of shoots was produced in MS medium supported with 10 mg L^{-1} BAP at 20 and 30 days after culture (7.95 and 12.02 shoots/explants respectively). Further increase in concentration of BAP resulted in lower multiplication rate. However, explants which cultured in MS media with 1.0 mg L^{-1} NAA produce least number of shoots as described in Table-1.

Table-1. In-vitro effects of growth hormones on banana cv. Basrai at different intervals of days using shoot tip as an explant.

Trait	Days	Growth hormones		
		BAP	**NAA**	**BAP+NAA**
No. of leaves	10	4.15*	2.24*	5.67**
	20	6.46*	3.21*	7.39**
	30	9.57**	5.31*	10.67**
No. of shoots	10	4.22*	2.44**	6.47**
	20	7.95*	3.38*	8.33**
	30	12.02*	5.84*	13.00**
Shoot length (cm)	10	3.95**	2.31*	4.55**
	20	6.25**	3.97*	6.83**
	30	7.23**	4.21**	7.57**
No. of roots				
		IAA	IBA	IAA+IBA
	15	9.33**	1.00^{NS}	6.67**
	30	12.00**	1.33^{NS}	10.00**

NS = Non-significant (P>0.05); * = Significant (P<0.05); ** = highly significant (P<0.01)

Effect of BAP and NAA on shoot length

The results showed that BAP has significant effect on the shoot elongation as compared to NAA. Results showed that highest shoot length produced in MS medium supported with 7.5 mg L^{-1} BAP and 1.0 mg L^{-1} NAA at 10, 20 and 30 days after culture (4.55, 6.83 and 7.57 cm shoot length/explants respectively). Alone BAP also showing similar results as in combination with NAA (Table-1). The smallest shoots were produced in MS medium supported with 1.0 mg L^{-1} NAA at 10, 20 and 30 days after culture (2.31, 3.97 and 4.21cm shoot/explants respectively). These findings indicating that BAP is the most suitable growth hormone for increasing the shoot length of in-vitro grown banana cv. Basrai.

Effect of IAA and IBA on multiplication of roots

The results showed that IAA has significant effect on the number of roots as compared to IBA. Number of roots was greater in 0.5 mg L^{-1} concentration of IAA (9.33 and 12.00/explants produced at 15 and 30 days respectively). Second greater number of roots was produced in MS medium supported with 0.5 mg L^{-1} IAA and 0.5 mg L^{-1} IBA (6.67 and 10 roots/explants at 15 and 30 days respectively). The least no. of roots was produced in simple MS medium without any hormone (1.0 and 1.33 roots/explants produced at 15 and 30 days respectively). These results showing that in banana, IAA is the most effective hormone for the development of in-vitro roots.

Screening of BBTV free plantlets using ELISA

The presence of banana bunchy top virus that is responsible agent of banana bunchy top disease was investigated through ELISA on field grown banana plants. After complete hardening the plants were transferred in field under and allowed to grow under natural conditions. Sampling were done from field grown plants and tested against the BBTV by using ELISA. Total 30 plant samples were analyzed in triplicate fashion in ELISA plate and found that only three samples were positive while all the remaining samples were negative for BBTV (Table-2). Negative and positive control was also used in ELISA test for the comparison of in-vitro grown banana plant. In Table-2 A1, A2 and A3 are the positive control while B1, B2 and B3 are negative control for BBTV. The results in Table-2 indicating the samples C2, D1 and H12 are infected with BBTV while all other samples showing BBTV free response. This also indicated that the about 90% plants are free from BBTV.

Table-2. ELISA results for the screening of BBTV free banana plants.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.675	0.568	0.531	0.156	0.126	0.126	0.164	0.230	0.185	0.194	0.180	0.242
B	0.137	0.159	0.168	0.127	0.140	0.144	0.163	0.156	0.152	0.128	0.158	0.140
C	0.330	0.519	0.171	0.169	0.160	0.121	0.132	0.162	0.127	0.135	0.146	0.186
D	0.588	0.112	0.140	0.122	0.133	0.114	0.122	0.164	0.127	0.149	0.146	0.141
E	0.121	0.116	0.138	0.135	0.134	0.118	0.130	0.133	0.141	0.125	0.136	0.151
F	0.173	0.139	0.151	0.126	0.129	0.141	0.122	0.127	0.142	0.155	0.158	0.166
G	0.180	0.111	0.119	0.130	0.135	0.125	0.129	0.117	0.109	0.141	0.148	0.170
H	0.135	0.130	0.116	0.126	0.118	0.114	0.166	0.135	0.121	0.134	0.147	0.568

DISCUSSIONS

Banana is a chief source of carbohydrates, fiber and protein with minimum percentage of fat. Banana crop is affected by various types of insect/pests and diseases and drastically losing its yield potential annually. Among these, BBTV is the main cause of reduced yield all over the world including Pakistan. A study was planned to develop the BBTV free banana plants using in-vitro technology. The production of virus free banana using in-vitro approach is dependent on variety of factors including explants, composition of medium, method of culture, pH, temperature and time of incubation etc. [2]. Plant growth regulators are very essential for the in-vitro proliferation [1, 4]. Various concentrations of growth hormones in different combinations were practiced during in-vitro study. A number of parameters including number of leaves, number of developed shoots/plant, shoot length and number of roots was analyzed of in-vitro grown banana plants. In-vitro regeneration of banana plantlets is good source of production of virus-free plants against BBTV. The concentrations of BAP, NAA and IAA greatly influence the multiplication of banana plantlets. Maximum numbers of in-vitro shoots were observed on MS medium supplemented with 7.5 mg L^{-1} of BAP and 1.0 mg L^{-1} of NAA after 10 days of culture. It was also noted that alone BAP @ 10 mg L^{-1} produce maximum shoots after 20 and 30 days of culture. While 5.0 mg L^{-1} of BAP and 1.0 mg L^{-1} of NAA produce lowest numbers of shoots at 10, 20 and 30 days after culture. The concentrations and combinations of growth regulators greatly influence the efficiency of explants towards in-vitro multiplication of cells. This difference also may be due to genotype response towards growth hormones, culture conditions and also the combination of growth regulators. Likewise, the number of leaves was also maximum on MS supplemented with 10.0 mg L^{-1} of BAP after 10, 20 and 30 days of culture. There are many reports which also showed that alone BAP when used at higher levels produce more and healthy leaves as compared to other hormones [1, 14]. Highest shoot length was optimized using combination of BAP and NAA @ 10.0 mg L^{-1} and 2.0 mg L^{-1}, respectively. A number of auxins are being used to develop in-vitro roots in different tissue culture studies of various crops. Among these IAA, IBA and NAA are the most popular auxins which are actively used in various concentration in MS medium to develop the roots. In present research work IAA were used to generate in-vitro roots and found that MS supplemented with 0.5 mg L^{-1} of IAA is the best medium for the root development from in-vitro shoots. Different approaches are being employed to check the virus free plant including PCR by using specific primer set. Leaves from in-vitro developed potted plants were screened against BBTV using ELISA approach and found that more than 90% plantlets were resistant to BBTV. The resistant plantlets were further multiplied for mass scale production of disease free plants (Figure-1).

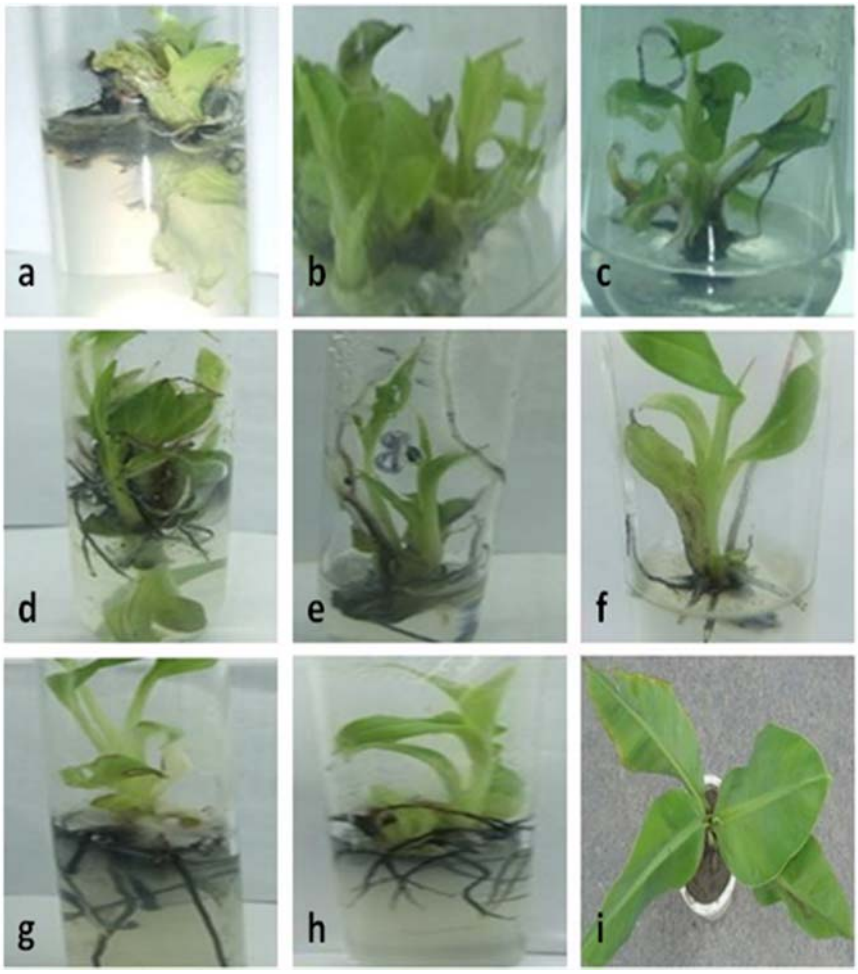

Figure-1. In-vitro regeneration, multiplication and rooting of banana Cv. Basrai using shoot tip as an explants and acclimatization of BBTD free plantlets in soil. (a) shoot tip explants on regeneration media (b, c) development of multiple shoots on regeneration media (d, e, f) elongation of in-vitro developed shoots (g, h) in-vitro development of roots (i) hardening of virus free plant under natural environment in soil.

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