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Effect of sterilants on shoot regeneration of Banana (*Musa acuminata*) cv. Monthan *in vitro* plantlets

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Abstract
In vitro culture of plant species most commonly depends on the optimal size of explants used for the purpose of study. Relatively, larger explants (3-10 mm) are desirable for rapid multiplication despite of its higher susceptibility to blackening and contamination. The surface sterilization of banana shoot-tips explants of desired size was performed before transferring them to the culture media to make our cultures contamination free. In the present study, different sizes of explants and different sterilants were used for determining the growth parameters of cv. Monthan and to check their ability of making a media contamination free. The toxicity percentage was seen to be dependent on the sterilants concentration and duration of time for being they are used. Double disinfection method was also used in case of longer persistence of contamination.

Keywords: contamination, *in vitro* culture, toxicity, sterilants, sterilization

Introduction
Banana occupies the status of commercial crop. Bananas and plantains are grown in about 130 countries around the world, exhibiting a spectacular production of 29124000 tons (FAOSTAT, 2016) [1]. Harvested area and Yield of Bananas was recorded as 846000 ha and 344255 hg/ha for Year 2016 (FAOSTAT, 2016) [1]. The banana is one of the oldest fruits known to mankind. From the nutritional point of view, bananas contain nutrients that boost energy levels and are good for heart and normal functionality of the muscles (PCARRD, 2007a) [11]. Expansion of banana production is frequently limited by costly high quality planting materials. The farmer-produced suckers are good transmitters of insect, pests and diseases (Rahman *et al.*, 2004) [14] and also suffer low multiplication* bulkiness, and poor phytosanitary quality (Vuylsteke, 1989) [17]. This has prompted interest in the use of *in vitro* tissue culture technique.
Various factors are known to influence the *in vitro* culture multiplication in banana. Investigating such factors would not only enhance the *in vitro* response but also help in accelerating the understanding of its basis. Optimum size of the explants varies with the purpose for which it is used. A relatively larger explant (3-10 mm) is desirable for rapid multiplication despite of its higher susceptibility to blackening and contamination. Minimal size of the explants increases the inoculation density in one culture vessel, cutting the production cost. During the micro propagation, the shoot and root growth appears to depend on explant density. The effect of inoculation density on plant growth is enhancing and has been reported in asparagus (Matsubara, 1973 and Matsubara and Clore, 1974) [5, 6] and potato (Sarkar *et al.*, 1997) [15].
For the production of 'clean' *in vitro* plantlets, field grown plants are indulged as a direct source of explants which brings a severe challenge in the form of microbial contamination during induction and establishment of viable *in vitro* cultures. The surface sterilization of shoot-tips explants was done before transferring them to the culture media to makes our cultures contamination free. Sodium hypochlorite is commonly used disinfectant for surface sterilization of banana explants (Muhammad *et al.*, 2004) [9], whereas some researchers have substituted with low concentration of mercuric chloride (Molla *et al.*, 2004 and Titov *et al*., 2006 [16, 8]. When the contamination persists for longer time in subsequent subcultures, double disinfection method is used (Rahman *et al.*, 2002 [13] and Madhulatha *et al*., 2004) [4].

During present investigation, *in vitro* shooting response in cv. Mon than affected by different explants size was examined to test the efficacy of explants size.

Materials and Methods

Plant material: The shoot tips situated on the upper most surface of sword suckers of rhizomes of banana plants covered by leaf sheath, was selected as source of explants. The suckers were washed under tap water to remove mud and other debris. Roots and outer leaf sheath were removed by a stainless steel knife in such a way that shoot tip was not damaged. The rhizomes were trimmed into the following sizes according to their length x breadth x height enclosing the shoot apex:

1. $E_1 = 1 \times 1 \times 1$ cm^3
2. $E_2 = E_1$ bisect vertically
3. $E_3 = 1 \times 1 \times 2$ cm^3
4. $E_4 = E_3$ bisect vertically
5. $E_5 = 2 \times 2 \times 2$ cm^3
6. $E_6 = E_1$ bisect vertically

Pretreatment of explants: The trimmed explants were pretreated with 1.0% w/v solution of Bavistin for an hour and washed in running water before surface sterilization. The explants were then kept in an antioxidant solution of citric acid and ascorbic acid each (100 mg/l) for one hour.

Surface sterilization of explants: The shoot was sterilized in 70 % ethanol for one minute and in 0.1 % w/v solution of $HgCl_2$ for 8 minutes. Finally, the explants were rinsed with double distilled water under the aseptic conditions to remove the traces of the above mentioned surface sterilizing agents. Explants were exposed or treated with different sterilants as listed in Table 1. These surface sterilized explants were inoulated to the MS medium containing 4 mg/l BAP and 2 mg/l Kinetin for shoot proliferation.

Table 1: Effect of explants on number of shoots formation, number of leaves formation and shoot length grown in Banana cv. Monthan

Treatments	Number of shoots after (days)				Number of leaves after (days)				Shoot length (cm) after (days)			
	7	14	21	28	7	14	21	28	7	14	21	28
E1	1.25	1.00	2.25	2.75	1.33	0.50	1.75	3.50	1.30	2.30	2.58	4.40
E2	1.00	1.75	1.75	2.50	1.00	0.25	1.00	2.75	0.17	1.72	2.65	3.58
E3	1.25	1.50	1.50	1.75	0.00	0.50	1.50	2.75	0.12	1.22	2.18	2.97
E4	1.25	1.50	1.50	1.50	0.00	0.25	1.75	2.75	0.03	0.97	1.85	2.55
E5	1.75	1.25	1.05	2.00	0.00	0.25	1.25	2.25	0.18	0.57	1.25	2.82
E6	1.00	1.25	1.05	1.25	0.00	0.25	0.75	1.75	0.38	0.05	0.87	1.87
SE (m)	0.308				0.735				0.798			
SE (d)	0.436				1.039				1.128			
C.V.	33.12				104.933				79.342			
C.D.	N/A				N/A				N/A			

Growth conditions: All the cultures were kept in growth chambers at 25±2^{0}C under 16 hr illuminations of cool white fluorescent tubes with a light intensity of 4000 lux. For regeneration, the cultures were placed under a 16-h photoperiod (4000 lux) provided by cool fluorescent tube lights.

Plant growth parameters: Banana explants were sampled after certain time interval of sterilants treatment after reaching an optimum stage of shoot proliferation and regeneration. Explants of each replicate and 3 replicates for each treatment were randomly selected for growth analysis. Number of shoots per explants, shoot length and number of leaves per shoot were measured after 7, 14, 21 and 28 days of inoculation to examine the effect of explants size.

Monthan explants were examined for survival percentage and percent toxicity, effected by different concentrations and durations of 3 sterilants used here i.e., Ethanol, Mercuric chloride and Bavistin.

Statistical analysis: The raw data obtained during the experimental observations were subjected to statistical analysis as per method by Gomez and Gomez, (1984) [2]**.** All measurements were evaluated for significance using analysis of variance (ANOVA) followed by OPSTAT and Duncan's Multiple Range Test (DMRT) test at the $P < 0.05$ level. All statistical analyses were conducted using SAS 9.2 (SAS Institute; Cary, NC, USA).

Results and Discussion

Plant Growth: The effects of explants size were monitored by measuring the changes in no. of shoots, shoot length, no. of leaves per shoot after 7, 14, 21 and 28 DOI. A factorial experimental design with a completely randomized arrangement was used. Table 2 shows that all the measured components of growth parameters were not significantly different for the main effects. Treatment E1 produced maximum number of shoot (2.75) followed by E2 (2.50), whereas E6 produced minimum number of shoots (1.25). E1 (1 x 1 x 1 cm^3) treatment also produced maximum no. of leaves (3.50) and highest shoot length (4.4 cm) followed by E2 and E3.

The explants optimal size depends on the purpose. Relatively larger explants (3-10 mm) are desirable for rapid multiplication despite of its higher susceptibility to blackening and contamination. The findings here suggest that as the size of the explants increases, the growth parameters of the genotype decreases. Thus, it is recommended that the size of the explants should be taken as minimal as possible to enhance the plant growth. During the micro propagation, the shoot and root growth appears to depend on explant density. The effect of inoculation density on plant growth is enhancing and has been reported in asparagus (Matsubara, 1973 and Matsubara and Clore, 1974) [5, 6] and potato (Sarkar *et al.*, 1997) [15].

Such effect can be seen due to some growth promoting substances diffusing from the explants. Similar results were reported by McClelland and Smith, 1990 [7]; Sarkar *et al.*, 1997 [15]; Prabhuling and Sathyanarayana, 2017 [12].

Table 2: Efficacy of different surface sterilants / disinfectants on culture survival percentage, percent toxicity, phenolic browning and plant vigour in banana genotype Monthan after 4-weeks of explant inoculation on culture media

Sterilants	Concentration (%)	Time (min.)	Culture survival percentage	Percent toxicity in culture	Phenolic browning	Plant vigour *
S1	70	0.5	0.0±0.00 ^{f}	0.0±0.00 ^{h}	Present	++
S2	70	1.0	0.0±0.00 ^{f}	0.0±0.00 ^{h}	Present	++
S3	0.1	5	28.0±2.00 ^{d}	50.3±1.52 ^{ef}	Present	+++
S4	0.1	6	74.0±1.00 ^{b}	25.0±1.00 ^{g}	Present	++++
S5	0.1	8	76.3±1.52 ^{b}	27.0±1.00 ^{g}	Present	++++
S6	0.2	5	25.0±2.00 ^{e}	76.6±1.52 ^{b}	Present	+++
S7	0.2	6	27.3±2.08 ^{de}	75.0±2.00 ^{b}	Present	+++
[S8	0.2	8	0.0±0.00 ^{f}	98.6±1.52 ^{a}	Present	++
S9	1.0	30	25.6±3.05 ^{de}	53.3±1.15 ^{cd}	Present	+++
S10	1.0	60	50.3±1.52 ^{c}	50.0±0.00 ^{f}	Present	+++
S11	1 : 1	6	75.6±1.52 ^{b}	55.3±1.52 ^{c}	Present	++++
S12	1 : 1	8	98.3±152 ^{a}	52.3±2.08 ^{de}	Present	++++

*Here, +++, ++ and + signs are indicating good, moderate and poor plant vigour. Data represents mean±SE of three replicates per treatment in three repeated experiments. Means within the same column followed the different letters are significantly different according to DMRT at 5% level.

Survival and toxicity percentage: Treatment of explants with 70% absolute alcohol was not effective and explants remained contaminated and therefore discarded. Shoot tip explants were sterilized using mercuric chloride and Bavistin at different concentrations and for different time intervals. It was observed that most of contaminants appeared during first week of inoculation of explants to the culture medium, area of which increased later with the lapse of time. The percent toxicity of the explants was concentration and time dependent i.e. it increased with concentration of sterilizing agent and the period of sterilization. Results differ significantly for the different sterilants used. Mercuric chloride was found effective in removing contamination from Monthan explants. Exposure of shoot tips explants to 0.1% $HgCl_2$ for 8 min. (S5) gave satisfactory removal of bacterial and fungal contamination followed (Table 3). Toxicity percentage increased to 98% without any survival of the explants with increase in the concentration and time duration of $HgCl_2$ (S8). Since, the infection was also observed during the subculture and after 4 weeks, an attempt was made to remove infection from shoot tips explants by pre-treatment of explants with bavistin (1%) for 1 hour and subsequent sterilization with $HgCl_2$ (0.1%) for 8 min. 98.3% culture survival and 52.3% toxicity level was recorded in Monthan explants (Table 3). For the production of 'clean' *in vitro* plantlets, field grown plants are indulged as a direct source of explants which brings a severe challenge in the form of microbial contamination during induction and establishment of viable *in vitro* cultures. The toxicity percentage was dependent on time and concentration. The sterilants toxicity percentage increases with the increase in incubation time in the culture medium. Sodium hypochlorite is commonly used disinfectant for surface sterilization of banana explants (Muhammad *et al.*, 2004) [9], whereas some researchers have substituted it with low concentration of mercuric chloride (Molla *et al.,* 2004 and Titov *et al.,* 2006) [8, 16]. When the contamination persists for longer time in subsequent subcultures, double disinfection method is used (Rahman *et al*., 2002 and Madhulatha *et al*., 2004) [13, 4]. Sterilant Bavistin (1.0%) + Mercuric Chloride (0.1%) in 1:1 for 8 min showed highest culture survival rate, whereas highest toxicity percentage was recorded in 0.2 % Mercuric chloride for 8 min. Similar results were reported by various investigators (Onuoha *et al.,*2011; Goswami and Handique 2013; Yadav *et al.,* 2017) [10, 13, 18].

Conclusion

Banana production has been seriously affected in recent years by various fungal and viral diseases and by the exchange of plant propagation material from country to country or continent that involves the possible dissemination of diseases. Tissue culture (often micropropagation) is considered as an important technology for developing countries for the fabrication of disease-free, high quality planting material and the rapid production of many uniform plants. On the basis of our findings, useful conclusions, both having elementary and registered values can be made. Explants of size 1 x 1 x 1 cm^3 were found to be the most suitable for plant regeneration in banana cultivar cv. Monthan. Disease–free plant generation requires surface sterilization of explants before inoculation. Pretreatment of explants with bavistin (1%) for 1 hour and subsequent sterilization with 0.1% $HgCl_2$ for 8 min. was performed to remove infection from shoot-tips explants whereas. 98.3% culture survival and 52.3% toxicity level was recorded in cv. Monthan explants.

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