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Standardization of *in vitro* Regeneration Technique in Elephant Foot Yam (*Amorphophallus paeoniifolius* L.)

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A B S T R A C T

The present investigation was carried out to standardize surface sterilization and culture establishment technique for *in vitro* regeneration of elephant foot yam. The well-developed apical buds from the corms of variety Gajendra were used as explant. Among various surface sterilization treatments tried, the treatment T_5 i.e., 750 mg/L Carbendazim (overnight) before cut, 12.5 ml/L Sodium hypochlorite (20 min), 10 ml/L Tween 20 (15 min), 10 ml/L Dettol and 50 ml/L Savlon (30 min), 1 g/L Carbendazim (10 min), under running warm water (1 hr), 2 g/L Carbendazim (15 min), 70 % Ethanol (2 min), 0.12 g/L Mercuric Chloride (15 min), 50 ml/L Sodium hypochlorite (5 min), 16 ml/L GA_3 (10 min), + 3 ml/L Streptomycin (20 min), 750 mg/L Cefotaxime (I) (20 min) and 750 mg/L Cefotaxime (II) (40 min) was found best combination for achieving the highest percentage of aseptic cultures (88.57%) while, the treatment T_2 recorded lowest (20%) establishment of aseptic culture. Among the various combinations, MS medium supplemented with 6 mg/L BAP + 0.5 mg/L NAA showed highest percentage of culture establishment (60%). The media combination of MS + 6 mg/L BAP + 0.5 mg/L NAA + 0.1 g/L Ascorbic acid recorded the highest percentage (88.89%) of shoot induction. Also, it showed minimum (15.33) days for shoot induction. The same media combination found effective for the highest average number of multiple shoot bud induction (5.33).

Introduction

Elephant foot yam (*Amorphophallus paeoniifolius* L.), belonging to the family Araceae, is one of the important perennial herbaceous tuber crops distributed in tropical and subtropical regions. Owing to its culinary properties, therapeutic values, medicinal utility and higher yield potential, it is referred as "the King of tuber crops". It is widely

cultivated as a cash crop due to its production popularity and potential as a tuberous vegetable. Elephant foot yam has great scope for commercial exploitation in the food industry because of its functional properties and nutritional components (Chattopadhyay *et al.,* 2009). It has antioxidative, hepatoprotective and uterus stimulating effect (Singh *et al.,* 2011). Gajendra is one of the popular varieties of elephant foot yam commonly cultivated in Konkan region of Maharashtra. This variety is well-known for its high yield, blackish brown colour corm and light yellow coloured flesh.

There are many species of elephant foot yam plant in the South East Asia i.e., *A. konjac*, *A. rivierii*, *A. bulbifer* and *A. oncophyllus.* Total area under cultivation of elephant foot yam is about 35 thousand hectares and production is about 917 thousand tonnes with productivity of 26.2 tonnes per hectare in India.

In the traditional propagation of elephant foot yam, tubers are generally used for multiplication. As a result, about 20% of the harvest per year is utilised as the seed material for the next season and is considered a costly affair (Asokan *et al.,* 1984). Also, the multiplication rate is very low and season bound. In addition to this, vegetative method always leads to risk of catching diseases. Lack of sufficient seed material of uniform size and dormancy is one of the major constraints limiting the production of elephant foot yam (Shirly *et al.,* 2013). Hence, the production of this crop is observed in a limited area of the Konkan region.

The micro-propagation techniques in elephant foot yam will facilitate in producing large quantities of elite planting material. Among the series of procedures required in *in vitro* propagation; appropriate explant selection, effective explant sterilization and culture establishment are most critical and important steps for successful micropropagation. This will ultimately help in overcoming the problems associated with the conventional propagation method. The application of tissue culture technique is useful to produce virus and disease free plants throughout the year.

Materials and Methods

The present investigation was carried out during the academic year 2019-2020 in Tissue Culture Laboratory of Plant Biotechnology Centre, College of Agriculture, Dapoli, Dist: Ratnagiri (M.S.), on elephant foot yam. The healthy and disease free corm explants were used from variety *viz.* Gajendra, obtained from "Central Experiment Station, Wakawali" Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli (M.S.). The experiment was conducted in completely randomized design (CRD) with three replications.

Explant preparation and sterilization

Healthy corms possessing well-developed apical buds and lateral buds were selected. Initially, these corms were brushed carefully to remove dust particles and washed thoroughly under running tap water. Several steps were followed to prepare number of explant by cutting the corms into small pieces. The explant was cut to a final size of 3 cm^3 (after 4-5 cuts).

The cuts were made in such a way that each explant contained at least one bud. Every cut was done by surface sterilization with different sterilization agents for varied time period and was followed by washing with double distilled water for 3-4 times to remove chemical traces as given in Table 1.

Culture medium

The basal medium developed by Murashige and Skoog (1962) was used with permutation

and combinations of plant growth substances. Thereafter, pH adjusted to 5.8 and 0.8% agar was added in semisolid medium prior to autoclaving. After autoclaving the culture media bottles were stored for further use.

Inoculation and incubation of explant

After sterilization the explants were inoculated on culture establishment media containing MS medium supplemented with various concentrations of PGRs. Initially, the cultures were incubated up to 30 days on the establishment medium. Then the established culture was transferred for shoot initiation and multiplication. Explants were sub-cultured after every 15 days on new media containing same media combination for multiplication.

Experimental conditions

All the procedure was carried out aseptically in laminar air flow chamber. The standard sterilization technique was followed as suggested by Street (1977). The experiments were conducted under well-defined conditions of culture room maintained at 25±2˚C temperature, uniform light (1600Lux) provided by fluorescent tubes (7200 K) over a light and cycle of 16/8 hours in day.

Tables of experimental results

Results and Discussion

Maximum aseptic cultures with minimum mortality are mostly desired. Therefore, to obtain aseptic culture it is necessary to treat the explants with various sterilizing agents. During surface sterilization, the concentration, time of exposure and combination of sterilizing agents were optimized.

Effect of surface sterilization treatment on percent aseptic culture

The observations of surface sterilization treatment on percent aseptic culture are presented in Table 1. It is evident that production of aseptic cultures ranged from 20 to 88.57 percent. Out of various treatment combinations tried, the treatment T_5 recorded the highest percentage of aseptic cultures (88.57%) followed by Treatment T_4 (57.75%) and T_3 (50%) whereas the treatment T_2 recorded the lowest 20% establishment of aseptic culture.

In the present study, the per cent aseptic culture given in Table 1 revealed that the treatment combination T_5 involving of 750 mg/L Carbendazim for overnight (Before cut) + 12.5 ml/L Sodium hypochlorite for 20 min + 10 ml/L Tween 20 for 15 min + 10+50 ml/L Dettol and Savlon for 30 min + 1 g/L Carbendazim for 10 min + under running warm water for 1 hr. + 2 g/L Carbendazim for 15 min + 70% Ethanol for 2 min + 0.12 g/L Mercuric chloride for 15 min + 50 ml/L Sodium hypochlorite for 5 min + 16 ml/l GA_3 for 10 min + 3 ml/L Streptomycin for 20 min + 750 mg/L Cefotaxime (I) for 20 min + 750 mg/L Cefotaxime (II) for 40 min was found to be the best combination to achieve highest percentage of aseptic culture (88.57%).

The per cent aseptic culture (88.57%) obtained in this study was greater than that obtained by Kamala and Makeshkumar (2014). In their study, they reported 70% aseptic culture using slightly similar surface sterilization treatment for corm bud explants of elephant foot yam. Also, similar surface sterilization treatment was used by Koli *et al.,* (2014) in banana for the sucker explants.

Table.1 Effect of sterilization treatments on per cent aseptic culture

Treatments	Sterilizing agents	Concentration	Exposure time	Per cent aseptic culture
T_1	Carbendazim (Before cut)	2 g/l	6 hrs.	0.00 (00)
	Thiophen methyl	0.2 %	Overnight	
	Sodium hypochlorite	10%	20 min.	
	Carbendazim	1 g	35 min.	
	Sodium hypochlorite	10%	15 min.	
	Carbendazim	1 g	25 min.	
	Tween 20	5%	15 min.	
	Dettol+Savlon	5 ml+ 45 ml	35 min.	
	Running tap water	-	1 hr.	
	Treatment in Laminar Air Flow Hood			
	Carbendazim	0.5 g/l	20 min.	
	Ethanol	70%	2 min.	
	Mercuric chloride	5%	15 min.	
	Streptomycin (1)	16ml/l	10 min.	
	Streptomycin (2)	20 ml/l	15 min.	
	Cefotaxime (1)	750 mg/l	25 min.	
	Cefotaxime (2)	750 mg/l	45 min.	
T_2	Carbendazim (Before cut)	1 g/l	Overnight	20 (26.57)
	Sodium hypochlorite	12.5 ml/l	20 min.	
	Carbendazim	1 g/l	30 min.	
	Tween 20	10 ml /l	15 min.	
	Dettol+Savlon	10 ml+50 ml/l	30 min.	
	Running warm water	45-50℃	1 hr.	
	Treatment in Laminar Air Flow Hood			
	Carbendazim	0.5 g/l	30 min.	
	Ethanol	70%	1 min.	
	Mercuric Chloride	0.12 g/l	20 min.	

	Sodium Hypochlorite	60 ml/l	10 min.	
	GA_3	16 ml/l	10 min.	
	Streptomycin	3 ml/l	20 min.	
	Cefotaxime (1)	750 mg/l	20 min.	
	Cefotaxime (2)	750 mg/l	40 min.	
T_3	Carbendazim (Before cut)	1 g/l	Overnight	50 (44.92)
	Sodium Hypochlorite	12.5 ml/l	20 min.	
	Carbendazim	1 g/l	30 min.	
	Tween 20	10 ml/l	15 min.	
	Dettol+Savlon	10ml+50ml/l	30 min.	
	Running warm water	45-50℃	1 hr.	
	Treatment in Laminar Air Flow Hood			
	Carbendazim	1 g/ l	30 min.	
	Ethanol	70%	2 min.	
	Diathane M-45	2 g/l	30 min.	
	Mercuric Chloride	0.12 g/l	20 min.	
	Sodium Hypochlorite	60 ml/l	10 min.	
	GA_3	16 ml/l	10 min.	
	Streptomycin	3 ml/l	20 min.	
	Cefotaxime (1)	750 mg/l	20 min.	
	Cefotaxime (2)	750 mg/l	40 min.	
T_4	Carbendazim (Before cut)	1 g/l	Overnight	57.75 (49.56)
	Sodium hypochlorite	12.5 ml/l	20 min.	
	Thiophene Methyl	1 g/l	30 min.	
	Tween 20	10 ml /l	15 min.	
	Dettol+Savlon	10 ml+50 ml/l	30 min.	
	Carbendazim	1 g/l	30 min.	
	Running warm water	45-50℃	1 hr.	
	Treatment in Laminar Air Flow Hood			
	Carbendazim	0.5 g/l	30 min.	
	Ethanol	70%	2 min.	

	DiatheneM-45	0.5 g/l	30 min.	
	Mercuric Chloride	0.12 g/l	20 min.	
	Sodium hypochlorite	50 ml/l	10 min.	
	GA_3	16 ml /l	10 min.	
	Streptomycin	3 ml/l	20 min.	
	Cefotaxime (1)	750 mg/l	20 min.	
	Cefotaxime (2)	3 ml/l	40 min.	
T_5	Carbendazim (Before cut)	750 mg/l	Overnight	88.57 (72.07)
	Sodium hypochlorite	12.5 ml/l	20 min.	
	Tween 20	10 ml/l	15 min.	
	Dettol+Savlon	10+50 ml/l	30 min.	
	Carbendazim	1 g/l	10 min.	
	Running Warm water	45-50℃	1 hr.	
	Treatment in Laminar Air Flow Hood			
	Carbendazim	2 g/l	15 min.	
	Ethanol	70 %	2 min.	
	Mercuric Chloride	0.12 g/l	15 min.	
	Sodium Hypochlorite	50 ml/l	5 min.	
	GA_3	16 ml/l	10 min.	
	Streptomycin	3 ml/l	20 min.	
	Cefotaxime (1)	750 mg/l	20 min.	
	Cefotaxime (2)	750 mg/l	40 min.	
SE(m) ±				**3.02**
CD at 1%				**13.54**

Table.2 Effect of media combination on establishment of explants

Tr. No.	Media Combinations	Per cent establishment
E_0	MS (Control)	0.00 (00)
E_1	MS + 4mgl^{-1} BAP + 0.5mgl^{-1} NAA	40 (38.94)
E_2	MS + 4.5mgl^{-1} BAP + 0.5mgl^{-1} NAA	20 (25.80)
E_3	MS + 5mgl^{-1} BAP + 0.5mgl^{-1} NAA	40 (39.04)
E_4	MS + 5.5mgl^{-1} BAP + 0.5mgl^{-1} NAA	40 (39.20)
E_5	MS + 6mgl^{-1} BAP + 0.5mgl^{-1} NAA	60 (51.32)
E_6	MS + 6.5mgl^{-1} BAP + 0.5mgl^{-1} NAA	46.66 (43.86)
E_7	MS + 7mgl^{-1} BAP + 0.5mgl^{-1} NAA	46.66 (43.91)
E_8	½MS	0.00 (00)
E_9	½MS +7.5mgl^{-1} BAP + 0.5mg^{-1} NAA	6.66 (18.90)
E_{10}	½MS + 8mgl^{-1} BAP + 0.5mg^{-1} NAA	10 (21.03)
E_{11}	½MS +8.5mgl^{-1} BAP+ 0.5mg^{-1} NAA	26.66 (30.97)
E_{12}	½MS + 9mgl^{-1} BAP + 0.5mg^{-1} NAA	20 (25.85)
E_{13}	½MS + 9.5mgl^{-1} BAP + 0.5mg^{-1} NAA	13.33 (22.20)
E_{14}	½MS + 10mgl^{-1} BAP + 0.5mg^{-1} NAA	36.66 (37.23)
E_{15}	½MS + 10.5mgl^{-1} BAP + 0.5mg^{-1} NAA	40 (39.06)
	SE(m) ±	**2.93**
	CD at 1%	**11.36**

Table.3 Effect of media combination on shoot induction and days to shooting

Tr. No.	Media combination	No. of days to shooting	Per cent Shooting	No. of shoot buds
S_0	MS (Control)	-	00 (00)	0.00
S_1	MS+4mgl^{-1} BAP+0.5mgl^{-1} NAA+ 0.1gl^{-1} Ascorbic acid	-	00 (00)	0.00
S_2	MS+4mgl^{-1} BAP + 1mgl^{-1} NAA+0.1gl^{-1} Ascorbic acid	23.67	22.22 (23.49)	1
S_3	MS+4mgl^{-1} BAP+1.5mgl^{-1} NAA+0.1gl^{-1} Ascorbic acid	20.67	33.33 (35.24)	2
S_4	MS+4.5mgl^{-1} BAP + 0.5mgl^{-1} NAA+0.1gl^{-1} Ascorbic acid	21.67	55.55 (48.22)	2.66
S_5	MS+4.5mgl^{-1} BAP +1 mgl^{-1} NAA+0.1gl^{-1} Ascorbic acid	17.33	77.78 (59.98)	3
S_6	MS+ 4.5mgl^{-1} BAP+1.5mgl^{-1} NAA+0.1gl^{-1} Ascorbic acid	16.67	66.67 (54.71)	4.33
S_7	MS+6mgl^{-1} BAP+0.5mgl^{-1} NAA+0.1gl^{-1} Ascorbic acid	15.33	88.89 (78.23)	5.33
S_8	½MS	-	00 (0.0)	0.00
S_9	MS+4mgl^{-1} BAP+0.5mgl^{-1} NAA+0.1gl^{-1} Ascorbic acid +0.1mgl^{-1} kinetin	23.33	11.11 (11.74)	3
S_{10}	MS+4mgl^{-1} BAP+ 1mgl^{-1} NAA+0.1gl^{-1} Ascorbic acid + 0.5mgl^{-1} TDZ	18.33	55.55 (48.22)	3.33
S_{11}	MS+4mgl^{-1} BAP +1.5mgl^{-1} NAA+ 0.1gl^{-1} Ascorbic acid + 0.1mgl^{-1} kinetin	21.67	22.22 (23.49)	2.33
S_{12}	MS+4.5mgl^{-1} BAP+0.5mgl^{-1} NAA+0.1gl^{-1} Ascorbic acid + 0.1mgl^{-1} kinetin	17.67	55..55 (48.22)	2.66
S_{13}	MS+ 4.5mgl^{-1} BAP+1mgl^{-1} NAA+0.1gl^{-1} Ascorbic acid + 0.5mgl^{-1} TDZ	19.33	66.67 (54.71)	3.33
S_{14}	MS+4.5mgl^{-1} BAP+1.5mgl^{-1} NAA+0.1gl^{-1} Ascorbic acid + 0.5gl^{-1} TDZ	20	44.44 (41.73)	3.66
S_{15}	MS+6mgl^{-1} BAP+0.5mgl^{-1} NAA+0.1gl^{-1} Ascorbic acid +0.1mgl^{-1} kinetin	18.33	55.55 (48.22)	4.33
	SE(m) ±		**7.98**	**0.35**
	CD at 1%		**30.46**	**1.36**

Plate.1 Plates of the experiment

A: Corm used as explant B: Sterile explants after cut used for inoculation C: Day 1 inoculated culture on establishment medium D: Established culture after 30 days E: First shoot initiation F: Shoot bud initiation on MS + 6mg/L BAP + 0.5mg/L NAA + 0.1g/L Ascorbic acid

In the process of *in vitro* regeneration, contamination is one of the major constraints as the corms, used as explants, are excised from the field.

Therefore, in the present study, Tween 20 utilized in the treatment T_5 proved to be effective as a surfactant to remove the surface contaminants like soil and dust.

Different antifungal reagents like Carbendazim, Diathane M-45 *etc.* and anti-bacterial reagents such as Cefotaxime and Streptomycin were effective for reducing fungal and bacterial contamination. Also, 70% Ethanol, Dettol and Savlon were used as disinfectants.

Effect of media combination on *in vitro* establishment of explant

Total 15 MS media combinations supplemented with BAP and NAA were used for *in vitro* establishment of explants. The recorded observations are presented in Table 2. The establishment percentage ranged from 6.66 to 60.00%. Amongst the different media combinations implemented, the highest culture establishment (60%) was obtained through the treatment E_5, which comprised of basal MS medium supplemented with 6 mg/L BAP + 0.5 mg/L NAA. The lowest culture establishment was observed in E_9 (6.66 %) containing ½MS + 7.5mg/L BAP + 0.5mg/L NAA. These results are in conformation with the findings of

Ahmed *et al.*, (2014). In his studies, using the suckers as the explant of Grand Naine cultivar of banana, he observed 63.33% culture establishment on MS medium containing 6 mg/L BAP.

Effect of media combination on shoot induction and days to shooting

The results obtained of per cent shoot induction and days to shooting are presented in Table 3. It was observed that the media combination S_7 comprising of MS + 6 mg/L BAP + 0.5 mg/L NAA + 0.1 g/L ascorbic acid showed highest percentage of shoot induction (88.89%) and reported minimum number of days for shooting (15.33 days). While, the lowest percentage of shoot induction was observed in treatment S_9 (11.11%) containing MS + 4mg/L BAP + 0.5 mg/L NAA + 0.1 g/L Ascorbic acid + 0.1 mg/L Kinetin and also, it required maximum number of days for shooting (23.67 days). From these results, it was revealed that, the shooting percentage increased with the increase in the concentration of BAP. Also, it was observed that the medium without any growth regulators, failed to show shoot induction. Similar results were obtained by Kamala and Makeshkumar (2014). In their study, they reported 95% shoot regeneration on MS basal medium supplemented with 5 mg/L BAP and 1.0 mg/L NAA. Also, Hu *et al.*, (2008) [4] reported the maximum shoot regeneration percentage (78%) in *Amorphophallus albus* on medium containing BAP and NAA in 4:1 ratio.

Effect of media combination on multiple shoot induction

The results obtained of multiple shoot bud induction are presented in Table 3. It was noticed that media combination (S_7) MS + 6 mg/L BAP + 0.5 mg/L NAA + 0.1 g/L Ascorbic acid was found to be superior amongst all other treatments producing maximum shoot buds (5.33) after second subculture. The lowest number of shoot buds (1) was recorded on treatment (S_2) containing MS + 4mg/L BAP + 1mg/L NAA + 0.1g/L Ascorbic acid. The single shoot was observed in each treatment after first subculture. In the *in vitro* multiplication of shoots, various growth regulators used and their concentration affect the multiplication rate. The media combination devoid of PGRs failed to show any shoot multiplication. These findings were in collaboration with the results given by Shirly *et al.*, (2013). They reported average of 5.7 shoot buds per culture on MS medium supplemented with BAP (6 µM) and NAA (0.5 µM).

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