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EFFECT OF PRE-DRYING, CEFOTAXIME AND SEASON ON *IN VITRO* CULTURE ESTABLISHMENT IN BANANA CV. KAMALAPUR RED (AAA)

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ABSTRACT
The present study was conducted for finding out factors affecting *in vitro* establishment of clean cultures in banana Cv. Kamlapur red. Healthy sword suckers were sterilized using sodium hypochlorite and mercuric chloride with a standardized protocol for culture initiation. The MS medium with 30gmL- sucrose, 8gmL- agar and 3mgL- BAP was used as culture medium. In the experiment on pre-drying of suckers under shade for 1-6 days after removal from the field, suckers pre-dried for five days showed significantly less contamination with highest clean cultures (76.40%), whereas without pre-drying showed more contamination and lowest clean cultures (43.17%). In the experiment conducted on effect of season of sucker collection, contamination was significantly less in suckers collected during summer season (63.40%) and however, suckers collected during winter were found to be best to dissect during culture initiation (60.45%). In the experiment on immersion of sucker in cefotaxime of 100-600mgL- of water for 15 minutes, suckers treated with 400 mgL- cefotaxime for 15 minutes showed significantly less contamination with establishment of highest clean cultures (85.62%). Suckers treated with both increased or reduced cefotaxime was not beneficial. The results of the present study are useful in obtaining clean cultures for mass multiplication.

INTRODUCTION

Bananas and plantains (*Musa* spp.) are important crops in the global fruit industry. Banana cv. Kamalapur Red (AAA) is a traditional variety grown in north Karnataka and has been registered under Geographical Indication Registry of India. Red banana plant grows to a height of 18 feet and crop duration is 18-20 months. Of late, this cultivar is becoming popular and its fruits have got huge demand due to the high nutrition, appealing taste and medicinal properties (cure of tuberculosis). Hence, production of large scale quality of planting material is need of the hour for saving and popularizing this traditional banana cultivar. Consequently, *in vitro* micropropagation is a biological technique for multiplication of large scale good quality planting material. Apart from the higher and faster multiplication rates, micropropagated banana plants have many advantages like regular availability of planting material, earliness, synchronized blooming and comparatively higher yields than conventionally propagated. Many research studies have been taken up towards standardizing *in vitro* multiplication (Kalimuthu *et al*., 2007; Anbazhagan *et al*., 2014; Radhika and Amutha, 2013; Prakash and Ramya, 2016) in several banana varieties belongs to AAA genome (Grand naine, Robusta, Dwarf Cavendish etc.). However, research studies for assessing factors affecting *in vitro* micro propagation of banana cv. Kamalapur Red have not been reported earlier except a study on effect of growth regulators by Uzaribara *et al*. (2015). Further, several studies in other banana cultivars have revealed that pre-drying of suckers (Sinha and Deka, 2016), antibiotic treatment (Grzebelus and Skop, 2014) and season of sucker collection (Dangi *et al*., 2009) has huge impact on establishment of contamination free clean cultures, which is first step in mass multiplication *in vitro*. Hence, an effort has been made for assessment of effect of pre-drying, cefotaxime and season on *in vitro* culture establishment in banana cv. Kamlapur red.

MATERIALS AND METHODS

Collection, sterilization and inoculation of explants

Sword suckers of banana cv. Kamalapur red weighing 0.50-0.75kg, from disease free, high yielding mother plant were collected and washed thoroughly using tap water to remove soil debris. Then removed outer layers of sucker to bring it to a size of 5-6 cm^2 using sharpen kinfe, disinfected with Bavistin (0.1%-60 mins) in 100 litre drum (make sure that suckers are immersed completely in the solution), later taken out into a beaker and treated with Mercuric chloride for 15 minutes (0.1%, prepared freshly in sterile water-Himedia). Then the suckers were collected into a fresh beaker, treated with Ascorbic acid (25mg**L**-, Himedia) and Citric acid (25mg**L**-, Himedia) solution for 30 minutes and decanted the solution. Then suckers were brought into a Laminar Air Flow Chamber, treated with Sodium hypochlorite (4%) for 15 minutes and decanted the solution. Later, treated with ethanol (70%) for

60 sec using stop watch and decanted the solution. Then suckers were washed six times with sterile water to remove traces of disinfection agents and inoculated into culture establishment medium.

Preparation of medium, consumables and sterilization

MS Medium (Murashige and Skoog, 1962) containing, 30gmL- sucrose was gelled with 8gmL- of tissue culture grade agar (Biolab india Pvt. Ltd, India). The MS medium was supplemented with 6-benzyl amino purines (BAP) at 4 mgL-. The pH of the medium was adjusted to 5.8±0.1, before autoclaving. Then, 30-35ml of medium was dispensed into pre sterilized 250ml glass bottles. Consumable and tools used in the process of micro-propagation were sealed in autoclavable polythene covers. Later, media, tools and consumables were sterilized using in steam sterilizer (Nat steel Pvt. Ltd., India) at 121ºC and 15lbs for 18 minutes. The culture bottles and consumables were maintained at 24 ± 1ºC under 16 hour cool white, fluorescent light.

The effect of pre drying

Suckers were pre-dried for 1-6 days before sterilization and used in culture establishment using the mentioned protocol and culture medium.

The effect of season of sucker collection

Suckers were collected during rainy/winter/summer months and used in culture establishment using the mentioned protocol and culture medium.

The effect of antibiotic

Suckers were treated with 100, 200, 300, 400, 500, 600 mg cefotaxime per liter of water for 15 minutes and used in culture establishment using the mentioned protocol and culture medium.

Observations and statistical analysis

Observations were recorded on health of explants and contamination (Bacteria/Fungus) once in a week up to five weeks of initiation. All the experiments were repeated three times. Data was analyzed using ANOVA for finding significance of treatments.

RESULTS AND DISCUSSION

For *in vitro* propagation of banana, bacterial contamination is a great problem. Although initially surface sterilization works, later on microbial contamination at the base of the explant is observed within 7 to 15 days after inoculation. Huge number of explants is destroyed in the culture due to endogenous bacteria (Hadiuzzaman *et al*., 2001; Titov *et al*., 2006). It appears, protocols for the micropropagation of banana cultivars are genotype depended (Kahia *et al*., 2015). In the present study, we have worked out few factors that reduce contamination and helps in establishment of clean cultures for subsequent plant multiplication of banana cv. Kamalapur Red.

The effect of pre-drying.

Stress induction in banana cv. Malbogh (AAB) suckers through pre-drying was an inexpensive and effective mode of microbial contamination reduction (Sinha and Deka, 2016). In the present study, explants (suckers) of banana cv. Kamalapur red (AAA) were pre-dried before inoculation stage, which responded exceptionally well for microbial contamination reduction. After inoculation onto the culture medium, the aseptic miniature suckers swell at base and developed green colour after two weeks of inoculation and in third week it formed shoot with swollen corm. On an average, the survived explants change its colour from creamy white to green within

Table 1: Effect of pre-drying of suckers before sterilization on contamination in initiated cultures of banana cv. Kamalapur Red

Sl. No.	Treatment (drying in shade after washing with water)	Cultures/ suckers	Clean usedculture (%)	Bacterial/Fungal contamination (%)	Remarks
1	0 days after removal	50	43.17	56.83	Bacteria is active
2	1 days after removal	50	52.75	47.25	Bacteria is active
3	2 days after removal	50	53.59	46.41	Bacterial activity is reducing
4	3 days after removal	50	69.58	30.42	Bacterial activity is reducing
5	4 days after removal	50	70.37	29.63	Bacterial activity is reducing
6	5 days after removal	50	76.40	23.60	Less bacteria/Best treatment
7	6 days after removal	50	50.55	49.45	Deterioration of sucker
	SEM	-	1.12	-	
	CD at 0.1%	-	3.26	-	

Table 2: Effect of season of sucker collection on contamination in initiated cultures of banana cv. Kamalapur Red

Sl. No	Season	No. of suckers initiated	Contaminated cultures (Bacteria+ Fungus)	Clean cultures	Remarks
1	July/Aug/Sept/Oct (Rainy)	50	67.09	32.90	Bacterial contamination is more
2	Nov/Dec/Jan/Feb (Winter)	50	39.55	60.45	Best season for sucker collection
3	March/April/May/June (Summer)	50	36.59	63.40	Difficult to lift/ hard to handle during dissecting
	SEM	-	-	2.79	
	CD (0.1%)	-	-	8.61	

Table 3: Effect of Cefotaxime on contamination in initiated cultures of banana cv. Kamalapur Red

Sl. No	Cefotaxime (mg/lit)	No. of suckers initiated	Contaminated cultures (Bacteria + Fungus)	Clean cultures	Remarks
1	100	50	46.55	53.44	Bacteria /treatment not effective/suckers healthy
2	200	50	38.30	61.69	Bacteria /treatment not effective/suckers healthy
3	300	50	33.94	66.05	Bacteria /treatment not effective/suckers healthy
4	400	50	14.37	85.62	Less bacteria/Treatment effective/suckers healthy
5	500	50	13.01	86.98	Less bacteria/Treatment effective/suckers healthy
6	600	50	11.65	88.34	Less bacteria/Treatment effective/suckers were brown
	SEM	-	-	1.89	
	CD (0.1%)	-	-	5.52	

Figure 1: A contamination free sucker of Banana cv. Kamalapur Red *in vitro*

Figure 2: A contaminated sucker of Banana cv. Kamalapur Red *in vitro*

Figure 2: Response of a clean culture of Banana cv. Kamalapur Red *in vitro*

12 to 15 days (Fig. 2). Likewise Cronauer and Krikorian (1984) and Wong (1986) also reported similar trends in shoot tip cultures of *Musa* spps. In the present study, pre-drying of suckers has significantly affected appearance of contamination and 5 days of drying of suckers was found to be the best which showed 76.40% clean cultures (Table 1). One of the reasons for reduced contamination by pre-drying may be reduced bacterial load in the sucker due to reduction in moisture on the surface as well as inside of it. The result of present experiment is in agreement with earlier studies (Sinha and Deka, 2016). Besides reducing contamination, this technique also has the advantage of stretching inoculation period after material collection. The protocol developed by Sinha and Deka (2016) includes 21 days of osmotic stress induction of banana suckers for reduction in contamination. But in the present study upto five day of pre-drying reduced contamination and after that health of tissue was started detoriating, which may be because of difference in genotype, age and size of suckers. Titov *et al*. (2006) has reported that antioxidant treatment was necessary to reduce phenolic secretion in banana shoot tip explants. However, in this study, there was no such phenolic secretion observed in pre-dried suckers.

The effect of season of sucker collection

The season of explant collection has been found to affect the survival of explants through effects on microbial contamination (Thomas and Ravindra, 1997). Further, Martini and Papafotiou (2013) has reported that the season of explant collection, apart from affecting contamination and browning, also had a direct effect on shoot production. Similarly, in the present study, the season of sucker collection has significantly affected the appearance of contamination in initiated cultures of banana cv. Kamalapur red. The contamination was significantly less

(63.40%) in suckers collected during summer season (Table 2) compared to suckers collected from rainy and winter. However, lifting of suckers from banana orchard was difficult during summer and suckers were very hard and hence, there was difficulty in excising them during initiation. Though the suckers collected during winter showed little more contamination than summer season (60.45%), they were found to be the best as they were smooth for dissection during initiation and easy to lift from orchard during collection. The contamination as well as time taken for its occurrence was very high and early in suckers collected from rainy season, respectively. The reasons for increased contamination in suckers collected during rainy season may be availability of sufficient moisture in the field that favors bacterial activity to the greater extent. Similarly, Dangi *et al*. (2009) has reported highest contamination in suckers collected in the rainy season. Furthermore, they have expressed that there was maximum percentage of culture establishment in summer months collected suckers, which might be due to less contamination as compared to in the months of June and July. The high contamination rate observed during the wet season may have resulted from low temperature and high soil moisture, which is conducive for the growth of fungi and bacteria. Furthermore, endogenous phenol level, in general remains low during summer months. In the present study also, browing of explants of summer season was low compared to the other two seasons. Similar observations were also reported earlier by Josekutty *et al*. (2003).

The effect of antibiotic

The major cause of the microbial contamination is insufficient sterilization of explants, growth media, working tools and operators' hands (Omamor *et al*., 2007). An incorporation of antibiotics and antifungal agents into the growth media of plant cultures has been reported to eliminate microbial contaminants (Habiba *et al*., 2002). Since plants do not have an immune system to antibiotics and as such many of the antibiotics, that are effective against bacteria, fungi, *and* phytoplasmas, are toxic to plants as well (Sinha and Deka, 2016). Knowledge of the effect of the antibiotics on both bacteria and the plants is essential for the recovery of healthy plants. There are many factors that should be observed prior to control of *in vitro* contaminants through the addition of antibiotics to the growth medium. These include the appropriate concentration, the form and spectrum of action of the bactericide, and the phytotoxic effect on cultivation (Oliveira and Scherwinski-Pereira, 2016). In the present study on effect of cefotaxime has significantly affected appearance of contamination in initiated cultures in banana cv. Kamalapur Red. The suckers treated with 400 mgL^- cefotaxime for 15 minutes (85.62%) was found to be the significantly best treatment towards contamination reduction and establishment of healthy cultures (Table 3, Fig. 1, 2 and 3). Nevertheless, higher concentration of cefotaxime (more than 400 mgL^-) could reduce the appearance of bacteria to a little extent only, which seems uneconomical. Similarly, addition of cefotaxime at 500 ppm to the culture medium reduced systemic bacterial Contamination (Dangi *et al*., 2009). During in vitro culture, it is also important that the selected antibiotic is effective against the contaminating bacteria without compromising the normal development of the plants. For this reason, it is essential to conduct tests to evaluate the phytotoxicity of the antibiotic on the explants (Grzebelus and Skop, 2014). In the present study, the suckers treated with highest concentration of cefotaxime (600mg/lit) showed browning and looked unhealthy. Also, treatment of suckers for up to 25-30 minutes with 400 mgL^- was found ineffective in phytotoxicity with little improvement in contamination reduction as well and further increase in time resulted in browning of suckers in the culture medium after inoculation (data not presented). Similarly, use of antibiotics reduced contamination in banana *in vitro* propagation (Msogoya *et al*., 2012; Oliveira and Scherwinski-Pereira, 2016).

One of the most commonly encountered problems in *in vitro* culture establishment is the contamination by microbial contaminants. The present study has revealed that five day of pre-drying of banana suckers and collection of suckers from winter season and treatment with 400mg/lit cefotaxime are effective in contamination reduction in *invitro* cultures of banana cv. Kamalapur Red. The results should help in establishment of clean cultures, which is the first step in large scale *in vitro* mass multiplication of quality panting material in banana cv. Kamalapur red.

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