RESEARCH ARTICLE

Assessment of Detergent, Fungicide, Antibiotics and other Factors on in vitro Regeneration and Development of Protocol for Conservation and Commercial Scale Multiplication of Banana (*Musa spp.*) Cultivars

D. P. Prakasha[1*], M. Abdul Kareem[1], Dileepkumar Masuthi[2], S. P. Preetham[1], S. R. Mulla[2] and K. M. Shivkumar[1]

[1]College of Horticulture, Sirsi 581401 3 KRCCH Arabhavi 5 HREC, Bijapur, India
[2]University of Horticultural Sciences, Bagalkot-Karnataka, India

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Corresponding Author:
D. P. Prakasha
prakash.dp@uhsbagalkot.edu.in

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ABSTRACT

In the present study an effort was made to establish an efficient multiplication protocol after experimenting very important factors affecting the efficiency of establishment of clean cultures. The MS medium containing PGR/s as per requirement was used for culture and sub culture. There was significant effect of various parameters tested on establishment of clean cultures in these varieties and most of these parameters resulted in highest clean cultures in Cv. Kamalapur Red and lowest clean cultures in Cv. Yelakki banana. Further, significant results were found in in vitro root induction in different sized plantlets and the highest and lowest average number of roots was recorded in 'A' grade plantlets of Grand naine (7.12) and Yelakki (2.18), respectively. There was 100 percent root induction. However, as the number of roots increased, the average root length decreased and vice versa was noticed in all the cultivars. Later, a protocol starting from explant sterilization to primary hardening was designed and evaluated for its efficiency. It showed 85.23%, 39.89% and 88.45% clean cultures and 2.5, 1.9 and 2.7 multiplication ratios in Cv. Grand Naine, Yelakki banana and Kamalapur Red, respectively. The hardened plantlets of all cultivars were subjected to molecular analysis using RAPD markers and the genetic fidelity was stable. The methodology of micro-propagation would be highly useful in commercial production of large scale quality planting material in Cv. Grand Naine (Commercial cultivar), Cv. Yelakki (AB, Neypoovan group, recalcitrant to multiply in vitro) and in vitro conservation of endangered Cv. Kamalapur Red.

Keywords: *Grand Naine, Red banana, Neypoovan, Genome group, Micropropagation, Protocol.*

INTRODUCTION

Banana (*Musa* spp.) belongs to the family *Musaceae* is the top most important fruit crop in India with highest production and it is a very important staple food source in the world. However, many fungal and viral diseases affect its cultivation and like panama wilt and BBT viruses are threat for both commercial cultivation as well as germplasm conservation. Nevertheless, to satisfy the global demand in the field of agriculture for producing more food crops and plant based medicine, it is most essential to conserve the agricultural, economical, rare and endangered plants [1]. Propagation of banana through suckers is seriously limited due to low multiplication rate, clonal degradation and the perils of spreading disastrous diseases. As a solution to this, *in vitro* propagation of bananas provides excellent advantages over traditional propagation material, including production of uniform and disease free plantlets, high multiplication rate and physiological uniformity. The availability of disease-free material all round the year, rapid dissemination of new plant materials throughout the world and uniformity of shoots with reduced cost are the other additional benefits of micropropagation [2]. Also, *in vitro* propagated banana plants give higher bunch weight, more

fingers and hands and less variability in fruit size and shape and 39% higher yield has been recorded from tissue cultured banana plants than plants obtained from sword suckers [3]. However, the efficiency of micropropagation system is affected mainly by the rate of multiplication which is protocol dependent. It has also been observed that banana multiplication rate is ability to establish contamination free cultures as well genotypic dependent [4]. Though abundant information on the micropropagation of banana is available for cultivars in India and abroad [5], however, little or no information is available on the comparative response of genotypes to a micropropagation system. But, propagation percentage and repeatability of the method are matters of concern which ultimately need a comprehensive, repeatable and applied method for a wide range of genotypes to facilitate disease free production of banana crop on commercial scale [6]. Further, for *in vitro* micropropagation of banana, bacterial contamination is a big problem. Although initially surface sterilization works, later on microbial contamination at the base of the explant appears within 7 to 15 days after inoculation. Huge numbers of explants are destroyed in the culture due to endogenous bacteria [7]. A few individual research studies have been taken up on factors affecting establishment of contamination free cultures such as predrying of suckers, season of sucker collection, antibiotics, etc in different cultivars of Banana [8-10]. The results obtained from the above workers revealed significant effect of these factors on establishment of contamination free cultures. Growth and organ differentiation were dependent on the medium support (gelling agent) and differed between cultivars [11] in tobacco. Contamination control was affected by sequence of combination of chemical sterilizing agents used in Cavendish banana [12] and cv. Matti [13] Several studies on effect of hormones on root induction have been carried out in banana [14] and no information is available on impact of size of shoot on rooting ability. However, it is learnt that, root induction is also affected by the shoot quality, donor age, and genetic origin [15] in other corps. In conclusion, research studies on developing a full length *in vitro* mass propagation system suitable for both commercial production and conservation in banana across the cultivars belongs to different genome groups in banana are lacking. Hence, in the present study, an effort was made to establish an efficient multiplication protocol after studying very important factors affecting the efficiency of establishment of clean cultures *viz*., sterilization of explant suckers and size of shoots lets on *in vitro* root induction (A, B and C size), etc., in Banana Cv. Grand Naine (AAA and commercial cultivar), Yelakki banana (AB, Neypoovan group, recalcitrant to multiply *in vitro*) and Kamalapur Red (AAA and geographical indicator and cultivar under threat).

MATERIAL AND METHODS

Preparation of consumables and culture medium:

Culture medium was prepared with major, minor salts and vitamins as per MS Medium [16] added 30gmL^{-} sucrose, gelled with 8mg/lit of tissue culture grade agar (Biolab India Pvt. Ltd, India) along with BAP 5 mg/lit and NAA 0.1 mg/lit, adjusted pH to5.8±0.1, 30-35 ml of which was dispensed into 250 ml bottle glass bottle, heat sterilized at 121^{o}C and 15 lbs for 18 minutes using autoclave (Nat steel Pvt. Ltd., India). The consumables viz., cotton, scalpel holder, blade, etc., water were also sterilized using autoclave.

Experiments on factors affecting establishment of contamination free cultures:

The procedure for culture initiation was followed as per [17]. The sword suckers of banana cv. KR, YB and GN weighing 0.50-0.75kg, 50-60 days old were collected from disease free, high yielding mother plant and used for culture initiation. Experiments were conducted with a required number of treatments and replications required for statistical analysis to assess Fungicide (1 mg/lit Bavistin for 30 minutes), Cetrimide (1 mg/lit for 30 mins), season of sucker collection (Nov-Feb), predrying (5 days), *in vitro* sterilization treatment [Mercuric chloride (0.1%-15 mins)+Sodium hypochlorite (4%, 15 mins)+ ethanol (70%-60 sec], cefotaxime (400mg/lit for 15 mins), concentration of solidifying agent (agar-6gm/lit) and grade (size) of shoots on *in vitro* root induction (A, B and C grade) in three cultivars.

Effect of size of shootlet on in vitro root induction:

Shootlets of A (4.0-6.0cm), B (2.5-4.0cm), C (<2.5cm) grade were inoculated into the mentioned culture media containing NAA 1mg/lit.

Evaluation of in vitro mass propagation system:

The treatments experimented earlier were pooled to form a protocol and assessed its efficiency (Fig.1) through eight subculture cycles in three varieties of banana.

Observations and statistical analysis:

Experiments were conducted employing completely randomized design (CRD) with sufficient number of replications and experiments were repeated three times for authentication. Observations were made every week up to four weeks of initiation and subculture of experiment. The pooled data was presented as per cent/centimeter/numbers along with standard error of mean. Data was analyzed using ANOVA for finding

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Table 1: Effect of various factors affecting establishment of contamination free cultures under *in vitro* micro-propagation of banana cultivars

	Fungi-cide[1]	Bacteri-cide[2]	Season (Nov-Feb)	Pre-drying (5days)	Steriliza-tion[3]	Cefotaxime (400mg/lit)	Agar (6gm/lit)
Kamalapur Red (KR)	46.31	52.07	61.37	76.05	77.11	85.63	2.52
Yelakki Banana (YB)	34.25	38.58	52.05	61.78	65.80	71.77	1.87
Grand Naine (GN)	48.09	53.80	64.38	73.37	73.56	83.19	2.66
S.Em±	0.481	0.369	0.545	0.469	0.565	0.516	0.011
CD @1%	1.482**	1.138**	1.681**	1.446**	1.743**	1.590**	0.035**

** Highly significant; [1] Bavistin@1mg/lit for 30 min; [2]Citrimide@1mg/lit for 30 min; [3] Mercuric chloride (0.1%-15 mins)+Sodium hypochlorite (4%, 15 mins)+ ethanol (70%-60 sec)

Table 2: Effect of size of shoot on *in vitro* root induction under *in vitro* micro-propagation of three banana cultivars

Size of shoot/ Variety	Root induction			No. of roots			Root length		
	KR	YB	GN	KR	YB	GN	KR	YB	GN
A grade	100	100	100	4.53	2.083	7.08	6.19	5.63	4.00
B grade	100	100	100	4.183	2.088	6.99	5.11	5.26	3.62
C grade	82.82	80.27	82.54	6.553	2.087	9.18	3.12	6.40	2.82
S. Em (±)			0.46			0.07			0.06
CD @1%			1.763**			0.265**			0.238**

** Highly significant; KR- Kamalapur Red; YB- Yelakki; GN- Grand Naine

significance of treatments.

Molecular analysis using RAPD markers for assessment of genetic fidelity:

The DNA from leaves of hardened micropropagated plantlets of the three varieties was isolated and PCR was carried out using eight random primers (OPA-02, OPA-9, OPA-19, OPB-06, OPC-03, OPB-15, OPD-11 and OPF-12) as per the protocol standardized by Shiddalingeswara *et al*. (2018) in banana. Gels were observed closely for differences in amplification pattern between mother and micro-propagated plants.

RESULTS AND DISCUSSION

Establishment of contamination free cultures is the most important step towards *in vitro* mass multiplication of a crop. Especially, sucker, as most used explant for *in vitro* multiplication of banana is collected from soil, intensive efforts are necessary for sterilization of explants to suppress contaminants. Explants taken from the external environment will leads to the mortality of the plant tissue due to microbial contamination [18]. In plant tissue culture, plants are grown in the nutrient media, where the nutrients required for the plants are added and also had a space for microbes, because of having rich nutrients. Nevertheless, microbial growth in the media is due to improper sterilization and ill procedures. Elimination and reduction of contaminants in *in vitro* culture caused by bacteria and fungi is an important key factor in obtaining aseptic explants [19]. NaOCl, $HgCl_2$ and antibiotics (cefotaxime, gentamicin, rifampcin) are general disinfectants that are applied to eliminate contaminants individually or in combination with others [20]. Further, contamination in tissue culture is not universal to find the exact reason but implies perfection in every step leads to reduce the contamination menace in plant tissue [21]. In earlier studies published from our laboratory, effect of pre-drying, antibiotics, season of sucker collection [22], sterilization treatment, growth regulators has [23] been assessed in Banana cv. Kamalapur red and effect of agar, adenine sulphate and light has been assessed in Banana cv. Grand Nine [24]. However, in most of the studies by other researchers, sterilization processes employed was unique, but effect of sterilization treatment on clean culture is not reported and they have concentrated mainly on growth regulators.

Effect of fungicide

Treatment of suckers with Bavistin (1 mgL^{-1}) for 30 minutes after washing thoroughly to remove soil debris, significantly reduced the fungal contamination. Banana Cv. Grand Naine recorded the highest clean cultures and Cv. Yelakki recorded the lowest clean cultures (Table1). Suckers soaked in fungicide solution (5gm/lit Bavistin) for 6 to 8 hours [25]recorded no fungal contamination in banana cv. Rasthali, where as [26] reported that treatment of suckers with fungicide solution (2 gm/l Bavistin) recorded least of 36% contamination in banana cv. Malbhog. Hence, though

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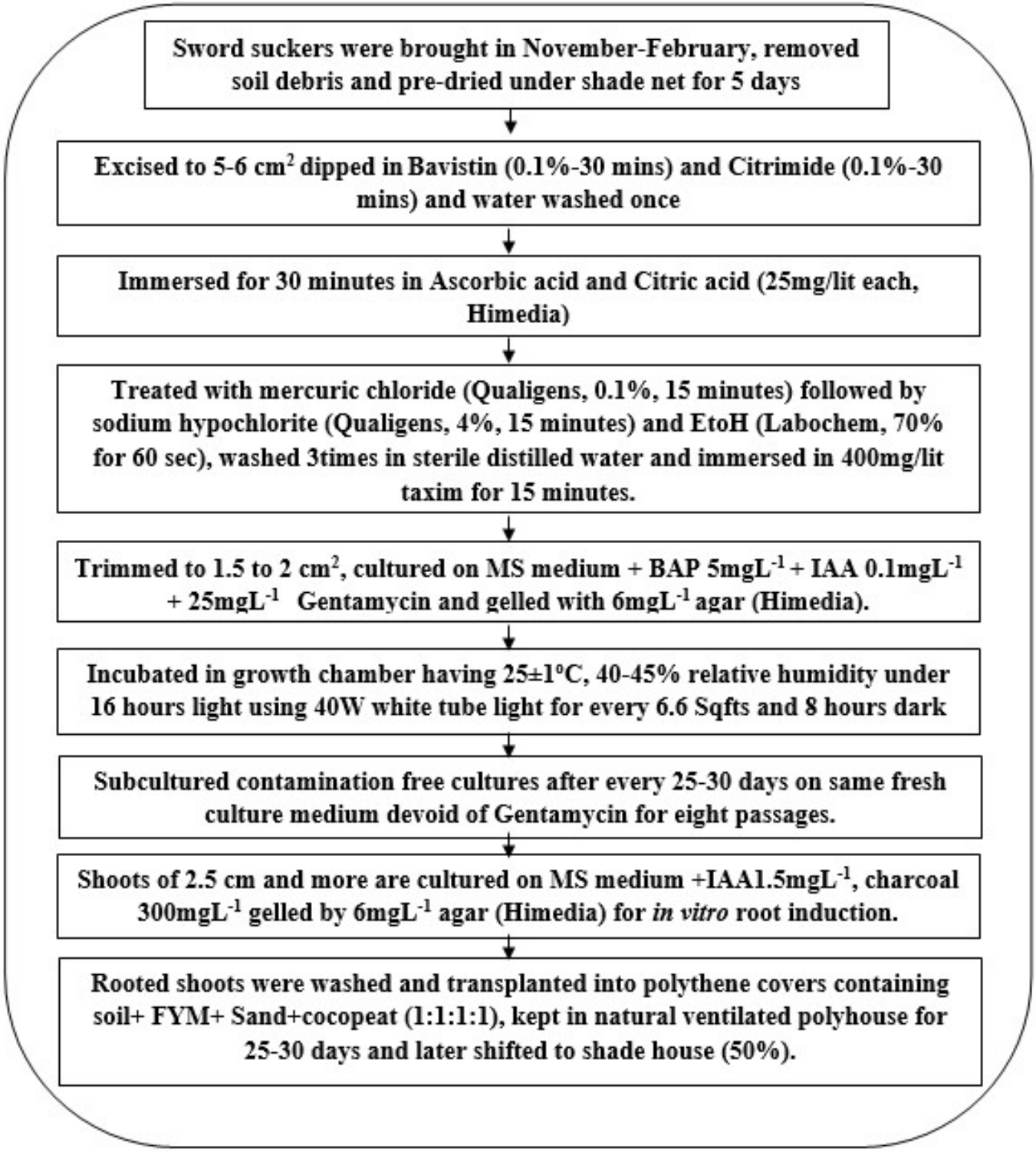

Fig.1: Flow chart of *in vitro* micro-propagation system evaluated for three banana cultivars.

contamination reduced, it varied with strength of fungicide solution and genotype.

Effect of Detergent

Treatment of suckers with a detergent (cetrimide, Quligens- 1 mgL^{-1}) for 30 minutes after fungicide treatment significantly the reduced contamination. Banana Cv. Grand Naine recorded the highest clean cultures and Cv. Yelakki recorded the lowest clean cultures (Table1). Cetrimide is a cationic surfactant, a quaternary ammonium derivative, which has demonstrated its effectiveness against gram-positive and gram-negative bacteria, also showing antifungal activity [27]. In earlier studies, washing with liquid detergent (Extran, Merck, 0.05 ml/l) [28], washing shoot tips with 0.05 % Citrimide for 30 minutes in banana cv. Rajapuri [29] and in Neypoovan (AB) [30] has been employed in sterilization process and impact has not been recorded. However, washing explants with cetrimide (1%) recorded 90 per cent clean culture establishments in *Centella asiatica* [31].

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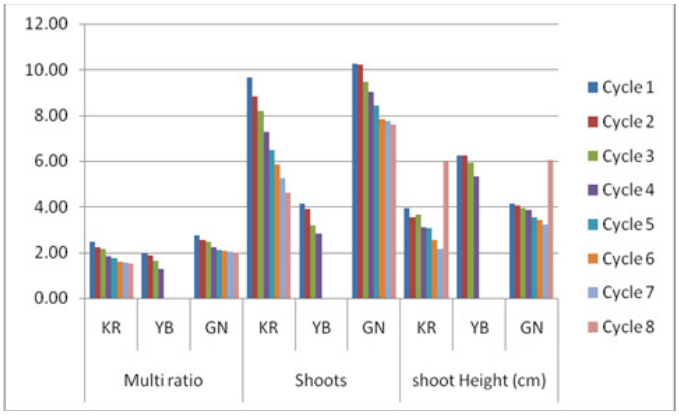

Fig. 2: Assessment of efficiency of *in vitro* micro-propagation system in three banana cultivars (KR-Kamalapur Red, YB – Yelakki banana, GN-Grand Naine)

orchard during collection [32]. Explants collected in March and April showed more browning than explants collected during any of the other months of the year in *Malosorbus* sp. [33]. Similarly, the season of explant collection has been reported to affect the survival of mango explants by affecting microbial contamination [34].

Effect of pre-drying

There was significant difference in contamination free cultures in three varieties due to pre-drying of suckers. Banana Cv. Kamalapur Red showed the highest clean cultures and Cv. Yelakki showed the lowest clean cultures (Table1). Similarly, pre-drying of suckers has

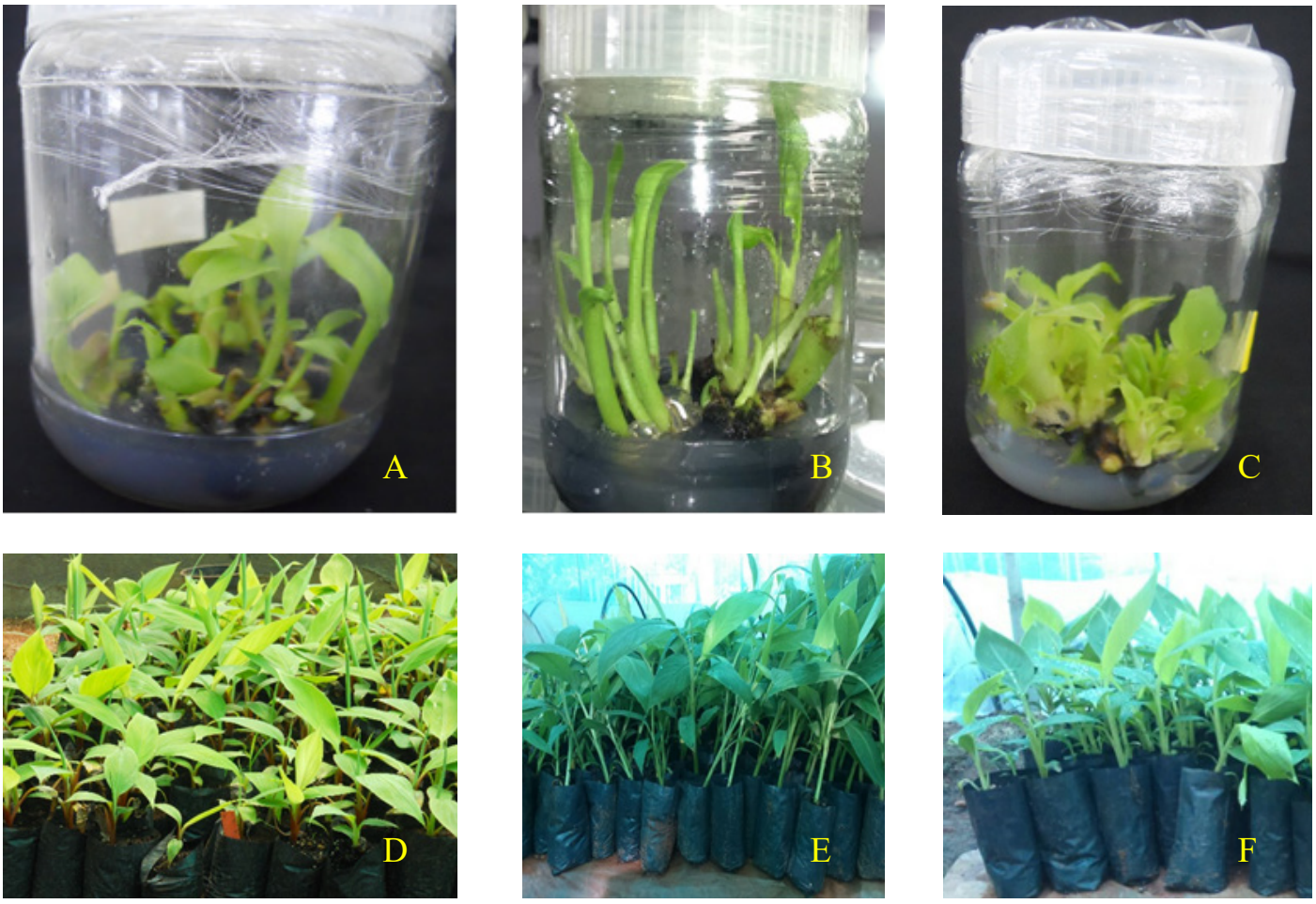

Fig. 3: *In vitro* shoot regeneration in Banana Cv. Kamalapur Red (3A), Yelakki (3B), Grand Naine (3C), and Hardened plantlets of Cv. Kamalapur Red (3D), Yelakki (3E), Grand Naine (3F)

Effect of season of sucker collection

There was significant difference in contamination free cultures in three varieties due to season of sucker collection. Banana Cv. Grand Naine showed the highest clean cultures and Cv. Yelakki showed the lowest clean cultures (Table1). Similarly, the suckers collected during winter showed less contamination than rainy season in banana Cv. Kamalapur Red and they were also found to be the best as they were smooth for dissection during initiation and easy to lift from

significantly affected appearance of contamination and 5 days of drying of suckers was found to be the best which showed 76.40% clean cultures [35]. One of the reasons for reduced contamination by pre-drying may be reduced bacterial load in and at the periphery of the sucker due to reduction in moisture on the surface as well as inside of it. Similarly, in the study by [36], increasing moisture loss has negative relation with microbial contamination. The lowest contamination (36%) was recorded in 21 days of shade storage where the sucker has lost 58% of moisture till 21 days.

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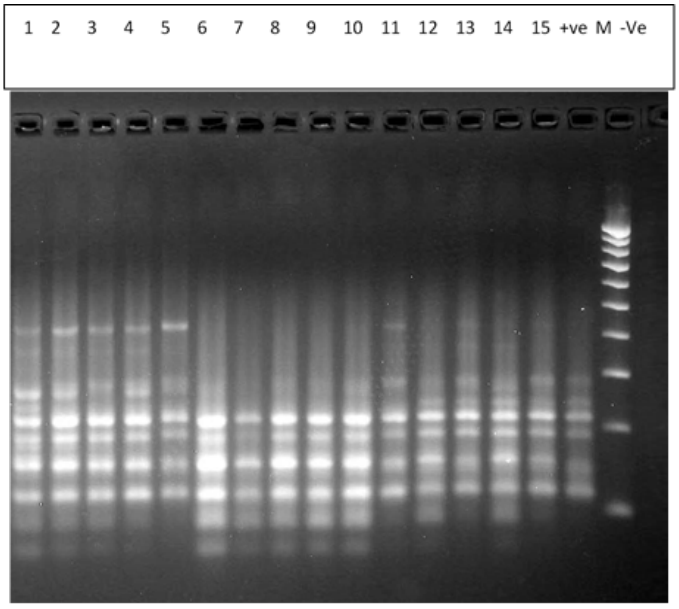

Fig. 4: Gel picture depicting genetic fidelity in hardened banana plantlets of Cv. Kamalapur Red (Lane 1-5), Yelakki (Lane 6-10), Grand Naine (Lane 11-15) using OPC-01 RAPD primer (+ve- positive control, M, 500 bp ladder with Bands from lower to higher-0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 kb, -Ve-Negative control)

Pre-drying appears as an inexpensive and effective mode of reducing microbial contamination in banana tissue culture. Apart from reduction in microbial contamination pre-drying also has provided time to plan for culture initiation after material procurement.

Effect of In vitro sterilization treatment

There was significantly reduced contamination in cultures of three varieties due to sterilization treatment [Mercuric chloride (0.1%-15 mins)+Sodium hypochlorite (4%, 15 mins)+ ethanol (70%-60 sec] of suckers. Banana Cv. Kamalapur Red showed the highest clean cultures and Cv. Yelakki showed the lowest clean cultures (Table1). In the other study, contamination varied from 20-90% with different levels and treatment time of these sterilizing agents in banana [37]. They recorded 90% contamination in suckers treated with Mercuric chloride (0.01%-2 mins)+Sodium hypochlorite (20%, 5 mins)+ ethanol (70%-60 sec) treatment which was reduced to 20% contamination upon treating with Mercuric chloride (0.01%-5 mins)+Sodium hypochlorite (40%, 8 mins)+ ethanol (70%-60 sec). It would be best evidence for reduction in contamination upon increased level of sterilizing agents to an optimum level. Further, suckers treated with absolute alcohol 95% for 30 second + sodium hypochlorite 1% for 10 min recorded 11% contamination in Bnanana cv. Matti [38]. Similarly, in cv. Grand Naine [29], in Cv. Red Banana[39] reduction in contamination has been reported with similar kind of sterilization process.

Effect of antibiotics

There was significant difference in contamination free cultures in three varieties due to treatment of suckers with Cefotaxime (400mg/lit) for 15 minutes. Banana Cv. Kamalapur Red showed the highest clean cultures and Cv. Yelakki showed the lowest clean cultures (Table1). Similarly, suckers treated with 400 mg/lit cefotaxime for 15 minutes showed reduced contamination (85.62%) in Banana cv. Kamalapur Red. [40] have stated that, 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. Further, in banana, [41] reported that an incorporation of antibiotics and antifungal agents into the growth media of plant cultures has been reported to eliminate microbial contaminants.

Cv. Yelakki (Ney Poovan) is not commercially propagated by the tissue culture industry because of the inherent problem of contamination in the initial establishment of the culture in the medium and the slow rate of multiplication [42]. Genotypes of *Musa* spp differed morphologically, biochemically, physiologically, pathologically and agronomically [22] which could be the reason for the difference in responses from the three cultivars to sterilization treatment in this study.

Effect of gelling agent (agar)

There was significant effect on multiplication ratio of cultures in three varieties due to gelling agent (agar-6mg/lit). Banana Cv. GN showed the highest multiplication ratio and Cv. Yelakki showed the lowest of it (Table1). The *in vitro* response of plantlets to gelling agent has been reported to depend on plant species [23]. [19] have reported that 6-6.5 mg/l was optimum to show high multiplication of 2.5 in 22-25 days with good outgrowth of cultures in Cv. Grand Naine. Further, effect of different gelling agents has been assessed and they recorded significant differences in banana Cv. Poovan [10]. These effects may be the result of differences in the strength of the gel produced, mineral composition and/or availability, and the presence of inhibitory compounds [39].

Grade (size) of shoots on in vitro root induction (A, B and C grade)

There was significant results recorded in vitro root induction in different sized plantlets. The highest and lowest average number of roots were observed in A/B grade plantlets of Cv. Grand Naine (7.12) and Cv. Yelakki (2.18), respectively (Table 2, Fig. 3-D,E,F). Hundred per cent plants showed root induction in all three cultivars. However, as the number of roots

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increased, the average root length decreased and *vice versa* was noticed in all the cultivars. Similarly, the *in vitro* developed shoots showed 100% rooting on MS medium supplemented with 4.92 micromolar Indole butyric acid (IBA) in banana (Sugandh and Kumar 2015). Various cultivars showed difference in their rooting parameters on the same media, which proves that the ability of rooting depends on genotype [13].

Efficiency of protocol

A multiplication system starting from explant sterilization to primary hardening was developed by combining with the optimum levels of parameters studied in the experiments and evaluated for its efficiency (Fig. 2; Fig. 3-A, B, C). It showed 85.23%, 39.89% and 88.45% clean cultures and 2.5, 1.9 and 2.7 multiplication ratios in Grand Naine, Yelakki Banana and Kamalapur Red Banana, respectively. The protocol could produce 10.26, 4.15 and 9.66 shoots per one multiplication ratio (one culture bottle) with an average shoot height of 4.15, 6.23 and 3.95cm, respectively. There was decrease in multiplication rate, shoot numbers and shoot vigour with increase in number of passages (Fig). Similarly, Muhammad *et al*. (2004) that reported shoot multiplication rate reduced from second subculture and one cultured shoot tip can produce 124.6±117.4 plants on the average after five sub culturing in banana cv. Basrai. [18] have reported the maximum differentiation of shoots (92.05%) with 16.75 numbers of shoots per culture in Banana cv. Malbhog. However, there was reduction in number of shoots in second and subsequent subcultures. [38] has reported the differentiation of more than seven shoots per shoot tip culture in Banana cv. Battisa. Further, [17, 19] has reported varied rate of *in vitro* multiplication in different banana genotypes of *Musa* sp. Hence, it appears that the rate of differentiation and multiplication differs with genotype under *in vitro* multiplication of banana. However, the present protocol works efficiently for establishment of clean cultures and shoot multiplication in the three cultivars.

Genetic fidelity analysis using RAPD markers

The RAPD markers analysis has showed that the regenerated and hardened plants (Fig. 3-G, H, I) through the protocol are genetically stable and true to type of mother plant in all the three cultivars (Fig. 4). Earlier, RAPD markers have been successfully employed for genetic fidelity testing in long term micropropagated banana Cv. 'Rasthali' (Silk-Musa AAB [3] and 'Grand Naine' (Cavendish-Musa AAA) [28].

CONCLUSION

The protocol or system developed for *in vitro* multiplication is an efficient tool for both production of planting material and commercial productions in banana Cv. Kamalapur Red, Yelakki Banana and Grand Naine, three important genotypes. However, there is need to improve shoot multiplication rate in Yelakki Banana (AB, Neypoovan group) in future studies.

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