STANDARDIZATION OF *IN VITRO* MALE BUD CULTURE IN BANANA *MUSA* (AA) 'KADALI'

by

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(2016-12-013)

THESIS

Submitted in partial fulfilment of the requirement for the degree of

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Faculty of Agriculture
Kerala Agricultural University

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2018

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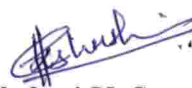
I, hereby declare that this thesis entitled **Standardization of *in vitro* male bud culture in banana *Musa* (AA) 'Kadali'** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, associateship, diploma, fellowship or other similar title, of any other University or Society.



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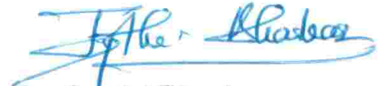

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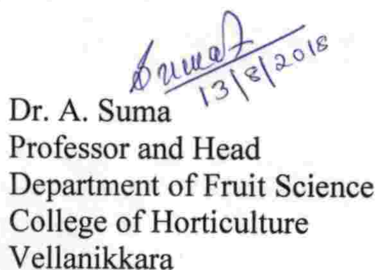

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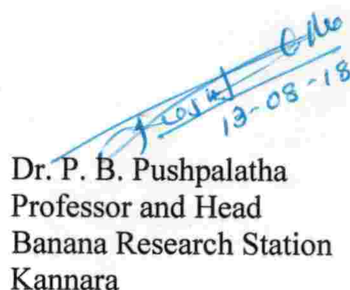

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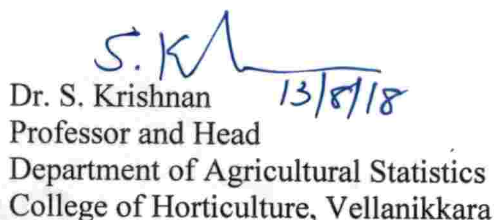

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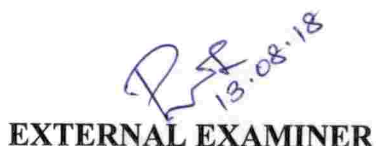

EXTERNAL EXAMINER

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ABBREVIATIONS

2 ip	2- isopentenyl adenine
2,4,5-T	2,4,5- Trichlorophenoxy acetic acid
2,4-D	2,4- Dichlorophenoxy acetic acid
BA	Benzyl adenine
BAP	Benzylaminopurine
CH	Caesin hydrolysate
CLB	Cauliflower like bodies
cv.	Cultivar
CW	Coconut water
HCl	Hydrochloric acid
$HgCl_2$	Mercuric chloride
IAA	Indole acetic acid
IBA	Indole butyric acid
MS	Murashige and Skoog medium
MT	Metric tonne
NAA	Naphthalene acetic acid
NaOH	Sodium hydroxide
PCPA	Pentachloro phenoxy acetic acid
TDZ	Thidiazuron
UV	Ultra violet
v/v	volume/volume
w/v	weight/volume

Introduction

1. INTRODUCTION

Banana is one of the world's most important fruit crop and constitute a staple food crop for millions of people in developing countries. World production of bananas and plantains is 1132.8 lakh MT, with India topping the list (291.24 lakh MT) with a total of 28 per cent of global production (FAOSTAT, 2016). Other major producers are Philippines, Ecuador, Indonesia, and Brazil. In tropics and subtropics, it is grown in all types of agricultural system, ranging from home steads to small farms and large commercial monocultures.

In India, a steady increase in area under banana was visible for the past four years but not with respect to production. Among different banana producing states, Kerala possess only the ninth position in area (1.29 lakh ha) and production (89.87 lakh MT) and sixteenth in productivity (15.02 MT/ha) (AGRISTAT, 2017). There is a huge gap between demand and production which is compensated by flow of fruits from neighbouring states.

One of the major limiting factors in increasing productivity of banana is the non availability of good quality planting materials through conventional propagation techniques. The availability of sucker which is the conventional planting material is limited due to the low production of suckers per plant. Likewise lack of availability of uniform suckers results in variation in flowering and fruiting leading to lack of synchronisation in harvesting thereby creating problem for the banana growers in harvesting and marketing their produce.

Micropropagation techniques enable the rapid multiplication of uniform disease free plants from a single plant with good genetic potential. In banana, tissue culture technique act as a strong platform to meet the huge demand of banana suckers throughout the country. The export of banana from our country is on increasing due to the regular supply of banana plantlets through tissue culture.

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In many of the commercial cultivars, *in vitro* shoot tip culture is being practiced (Kulkarni *et al.*, 2004) while, in a large number of local elite clones of banana, the protocol for producing plantlets through *in vitro* techniques is lacking. For commercial cultivation of banana in India, the most commonly used cultivars belongs to the triploid genomic groups. In Kerala also, triploid banana varieties are commercially cultivated. Nendran being the most important banana cultivar and other varieties of importance are Mysore Poovan, Red banana and Rasthali. The diploid varieties of importance in Kerala are Kadali, Njalipoovan and Kunnan. All these varieties are in great demand in the domestic market. Among these varieties, Kadali occupies a special place as a 'spiritual fruit' as it is used on a large scale for offering in temples and the fruits are bestowed with a special flavour besides possessing medicinal properties and therefore fetches premium price. However, the farmers are facing the problem of non availability of good quality planting material for commercial cultivation. The availability of tissue cultured plants of Kadali produced through shoot tip culture is meagre and the number of plants produced is not sufficient to meet the present day demand of banana farmers for the banana cultivar Kadali. As compared to other triploid bananas, regeneration of plantlets through shoot tip culture in Nivedyakadali is very low and also the problem of contamination of cultures is more (Sapheera, 2005).

Banana male bud can be used as a potential starting material for shoot regeneration to overcome all these hurdles (Krikorian *et al.*, 1993), as this part goes unutilized during the harvesting of banana bunches. Male flowers are protected inside the bracts on the male bud. Therefore, as compared to suckers, male buds are less affected by diseases and contamination rate is also less. As the availability of suckers are limited, male buds can also be used for raising tissue culture plants in addition to the suckers and one male bud can contribute to more number of explants. As male bud explants are less contaminated, simple sterilization treatments will be sufficient. Somaclonal variation and latent virus contamination are negligible in *in vitro* male bud culture (Harirah and Khalid, 2006). Selection of male bud from

bunches with desirable characteristics such as more number of hands and fruits per bunch is possible before culturing (Resmi and Nair, 2007).

In Kerala, low productivity due to lack of sufficient number of good quality planting material and the prevalence of sucker transmitted virus diseases *viz*., banana bunchy top, cucumber mosaic virus and banana bract mosaic limits the commercial cultivation of banana, which can be scaled up by developing a new vista for multiplying banana plants through *in vitro* techniques.

Developing a protocol for the mass multiplication of diploid banana clone *Musa* (AA) ' Kadali' through *in vitro* male bud culture will be of great help to farmers and the studies has to be conducted for developing a viable protocol. Keeping this in view the present study was taken up with the following objectives:

1. Standardization of establishment media for *Musa* (AA) 'Kadali'
2. Standardization of multiple shoot induction media
3. *In vitro* rooting and acclimatization

Review of Literature

2. Review of literature

Bananas are vegetatively propagated plants and generally seed setting is not observed. Conventionally bananas are propagated through suckers. The major limitation of conventional propagation of *Musa* cultivar is the low rate of multiplication of suckers (Vuylsteke and De Langhe, 1985). Apart from slow multiplication rate, spread of pathogens through vegetatively propagated banana suckers causes significant losses (Cronauer and Krikorian, 1985).

The concept of cell totipotency (Haberlandt, 1902) derived from cell theory (Schleiden, 1838) forms the basis of tissue culture. Micropropagation technique has been standardized in about 332 crop species (Vasil *et al*., 1979). Nearly, 1051 crop species have undergone *in vitro* studies (George and Sherrington, 1984) and among that 94 are fruit crops including bananas.

Tissue culture technology is getting overwhelming response from the farming community in the country and abroad due to improved yield and quality thus revolutionising the horticultural industry. Tissue culture, a process of crop propagation in a test tube under controlled and sterile conditions, has made banana cultivation an attractive proposition for farmers.

Use of tissue culture banana plants is becoming popular among farmers, due to increased yield and disease resistance. Barriers usually encountered during conventional method of propagation like slow multiplication, bulkiness and poor quality of suckers lead to difficulties in germplasm handling of banana. Micropropagation is a vital technique used not only for overcoming the problems but also for multiplying newly bred clones (Vuylsteke and Ortiz, 1996).

Shoot tip is the most commonly used explant in banana tissue culture. But the availability of this explant is limited in elite genotypes and this problem can be overcome by using male buds as a potential explant. Added to this, good quality plants can be located after assessing the bunch characters and their further

multiplication can be done by male bud culture which normally exhibits low contamination rate (Resmi and Nair, 2007).

The review has been confined to the *in vitro* male bud culture of banana cultivar Kadali, the important aspects of which are dealt under five major heads.

1. Explant
2. Media for culture establishment and multiplication
3. Culture conditions
4. *In vitro* rooting
5. Hardening

2.1. Explant

Different types of explants have been used for regeneration of bananas such as shoot tip (Kulkarni *et al.*, 2004; Kulkarni *et al.*, 2006), proliferating meristems (Cronauer and Krikorian, 1983), zygotic embryos (Cronauer and Krikorian, 1988), female flowers (Grapin *et al.*, 1996) and male flowers (Resmi and Nair, 2007). Male bud was found to be a potential explant for rapid multiplication of *Musa* species through *in vitro* culture (Smitha *et al.*, 2014).

2.1.1. Male bud explant

Krikorian *et al.* (1993) reported the formation of multiple shoots which were directly induced from the active meristems of inflorescence apices in banana. The response of different *Musa* clones to inflorescence apex culture were assessed by Ganapathi *et al.* (1999); Sebastian and Mathew (2004); Resmi and Nair (2007); Darvari *et al.* (2010); Smitha *et al.* (2014).

The success or failure of the culture also depend on the size of the explant used. Darvari *et al.* (2010) reported that variation in the size of male flowers under each tiny bracts can influence the regeneration rate of culture. They evaluated the

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in vitro multiplication of banana clones, Berangan (AAA), Rasthali (AAB), Nangka (AAB) and Abu (ABB) using different sizes of male bud. Male inflorescences of 15, 20 and 25 mm length cultured in MS medium responded differently to the number of 'cauliflower like bodies' (CLBs). Male inflorescence of 20 mm length was found to produce more number of CLB clusters.

Male bud of 1-5 mm size with apical dome was used as explant in two banana cultivars, Sannachenkadali (AA) and Red banana (AAA) on MS medium supplemented with BA and IAA (Resmi and Nair, 2007). Effect of male bud size on shoot initiation in diploid *Musa acuminata* cultivars on MS medium supplemented with TDZ was studied by Smitha *et al.*, 2014. The response obtained from male bud with minimum size (4-5cm) was found to be promising and explant size greater than 5 cm exhibited lower rate of shoot initiation.

Ganapathi *et al.* (1999) reported the successful development of embryogenic callus from young male flower primodia. Rapid proliferation of embryogenic callus was observed on a medium with BA and IAA. The banana cultivar, Grand Naine (AAA) produced somatic embryos and white, translucent callus in cultures using young male flower explant (Escalant *et al.*, 1994).

Sultan *et al.* (2011) explored the possibility to identify the optimum developmental stage of immature male buds for regeneration in *Musa* sp. cv. Sabri (AAB) and identified that the male flowers of bract numbers between 24 to 26 were found to be the best for inducing callus in MS medium. Bakry *et al.* (2008) identified male flowers of bract number of 21 and 22 as the best developmental stage for callus induction through the haploid production of *Musa balbisiana* (BB).

2.1.2. Response of genotypes

The different genotypes of banana behave differently in tissue culture. Vuylsteke and De Langhe (1985) reported that the genotypes can influence the rate and type of proliferative growth. Though they observed higher proliferative growth

in B genome, ABB showed lower proliferation compared to AAB genome. Resmi and Nair (2007) stated that the difference in *in vitro* responses of diploid and triploid banana cultivars were due to the effect of hormonal combinations in MS medium.

The response of three cultivars belonging to AAB genome and AAA genome namely Nendran (AAB), Palayankodan (AAB) and Red banana (AAA) in tissue culture were studied by Bhaskar (1991). The time taken for culture establishment and percentage of cultures established were different in each cultivar.

Sapheera (2005) viewed that production of multiple shoots were lesser in diploid bananas as compared to triploid acuminata types. Ganapathi *et al.* (1999) studied the embryogenic response of young male flowers of different banana genotypes. Rasthali (AAB) exhibited good embryogenic response compared to Shreemanti (AAA), Basrai (AAA), Lokhandi (AAA) and Trikoni (AAA).

2.1.3. Season of explant collection

The season of collection of explant influences the performance of cultures in *in vitro* conditions. Maximum explant survival and minimum contamination of banana cultures were noticed when explants were collected during November to April (Bhaskar, 1991). Explant collection from field during March – April resulted in maximum survival percentage (Sundararasu, 2003). Sapheera (2005) reported least contamination rate and maximum explant survival when explants were collected from field during March- April and November- December.

2.1.4. Surface sterilization

When explants collected from the field were used for initiating cultures under *in vitro* conditions, the main problem encountered is the microbial contamination of the explants. Hence, surface sterilization of explants has to be carried out before inoculation.

Several sterilizing treatments were tried by different workers using different concentrations of sterilants with varying time duration to identify the best sterilizing

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treatment in *in vitro* culture of banana. Vartika *et al.* (2013) documented that the higher survival percentage and best aseptic cultures in *Musa* cv. Dwarf Cavendish was obtained when explants were treated with mercuric chloride (0.1 %) for five minutes followed by quick dip in ethanol. The minimum contamination percentage and maximum survival percentage of explants of banana cv. Grand Naine was obtained for the sterilization treatment using $HgCl_2$ (0.1 %) at 10 minutes and 2 minutes respectively (Yadav *et al.*, 2017).

Sterilization of inflorescence explants were done using mercuric chloride (0.2 %) solution for 10 minutes to 15 minutes (Rao *et al.*, 1982). Titov *et al.* (2006) reported that surface sterilization of banana inflorescence buds with $HgCl_2$ (0.1 %) for 6 minutes recorded maximum number of contamination free cultures. Bhaskar (1991) achieved least contamination of floral apex explants by rinsing the explants with absolute alcohol (95%) for 30seconds followed by mercuric chloride treatment (0.05 %) for 10 min. Sapheera (2005) tried several surface sterilants for different banana explants such as sword sucker, peeper sucker and eye bud. The best result was obtained for the combination of mercuric chloride (0.1 %), absolute alcohol (70 %) and antibiotic cefatoxime (0.01 %).

2.2. Media for culture establishment and multiplication

The growth and morphogenesis of plant tissue culture mainly depends on the composition of the medium. The most widely used medium for tissue culture is Murashige and Skoog (1962) medium. Chemical composition and physical form of the nutrient medium influences the success of *in vitro* cultures (Murashige, 1974).

2.2.1. Composition of medium

Murashige and Skoog (MS) mineral salt mixture is very suitable for banana and plantain tissue culture (Banerjee *et al.*, 1986). Several investigators reported the use of Smith and Murashige (1970) medium in which the salt composition is identical to that of MS except for additional phosphate (Ma and Shii, 1972; Hwang *et al.*, 1984). However, majority of the research workers used slightly modified MS

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medium, with alterations in the organic constituents *viz.*, carbon, vitamins, amino acids and growth regulator supplements. (Banerjee *et al.*, 1986).

Sucrose is the preferred carbon source used in tissue culture. Refined grocery sugar can be substituted for sucrose of analytical grade (Banerjee *et al.*, 1986). Although glycine an amino acid is not essential in cultures, it acts as a source of readily available nitrogen for the cultured tissues (George and Sherrington, 1984). Adding 15% (v/v) coconut water was found beneficial in inducing growth in banana shoot-tips (Krikorian and Cronauer, 1984).

Different levels of sucrose in rooting of banana culture was tried by Bhaskar (1991). Sucrose at 1.5 per cent concentration was found to be optimum for early initiation of roots and production of maximum number of roots. Hussein (2012) evaluated the effect of sucrose, inositol and white's organics on banana cultures in MS basal medium and found that sucrose at 3.0 per cent increased the plantlet fresh weight, while inositol and white's organics showed no significant effect.

2.2.2. Growth regulators

In *in vitro* propagation, plant growth regulators are inevitable for the manipulation of growth and development of explants. The pattern of development in culture is often determined by their concentration and ratio in the medium. Auxins and cytokinins are the two major groups of growth regulators used in tissue culture. The most commonly used auxins are IAA, IBA, NAA, 2,4-D, 2,4,5-T, PCPA and cytokinins are BA, 2 ip and kinetin. The type of buds induced were determined by the hormonal balance established among the growth regulators (Trujillo and Garcia, 1996). The recommended BAP concentration for *in vitro* propagation of *Musa* ranges from 8.9 - 22.2 μM (Crouch *et al.*, 1998).

Cytokinins play a major role in reducing the dominance of apical meristems and inducing axillary as well as adventitious shoots from meristematic explants (Madhulatha *et al.*, 2004). The high response of BA over the other cytokinins in inducing multiplication in shoot tip cultures has been reported in different cultivars

of banana (Wong 1986; Kulkarni *et al.*, 2004; Kumar *et al.*, 2005). For banana shoot tip proliferation 5.0 mgL^{-1} BA was found to be optimum but for cultivars which exhibits low proliferation rate when cultured even in this medium, can be modified by increasing the concentration of BA above the standard level to increase the proliferation rate (Vuylsteke and De Langhe, 1985).

Vuylsteke (1983) studied the efficiency of BA at different concentration in *in vitro* regeneration and multiplication of 11 *Musa* cultivars. They found that the cultivars did not show any varietal difference in their regenerative capacity. Whereas, BA at 10.0 μM induced more proliferative growth than BA at 1.0 μM. Ten fold increase in rapid multiplication *in vitro* was achieved in the media containing BA 100.0 μM than in the regeneration medium (BA 10.0 μM) .

The proliferation rate due to the addition of cytokinins on banana was studied by Arinaitwe *et al.* (1999). The results demonstrated that the cultivars responded well to different TDZ concentrations and was found to be more economical than other adenine based cytokinins. Buah *et al.* (2010) stated that the degree of efficiency of shooting depends upon the type of hormone and plantain cultivar. The concentration of BAP at 4.5 mgL^{-1} was found to be optimum for inducing maximum number of shoots in *Musa* sp. Oniaba and Apantu pa followed by kinetin and 2 ip.

The effect of benzylaminopurine (BAP) and thidiazuron (TDZ) on multiplication of *Musa* sp. Berangan Intan (AAA), Berangan (AAA), Rasthali (AAB), Nangka (AAB) and Baka Baling (ABB) were studied by Shirani *et al.* (2009). BAP at 22.2 μM and TDZ at 2.0 μM were found to be the most suitable levels of hormone for commercial micropropagation system.

Jafari *et al.* (2011) revealed that MS media supplemented with benzylaminopurine (BAP) favoured the bud formation in shoot cultures of *Musa acuminata* cv.Berangan. The bud formation during the initial stage was found to increase proportionally with the increase in BAP concentration, whereas in the proliferation stage, abnormality of shoots were found to decrease with decrease in

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concentration of BAP. MS media supplemented with BAP 4.0 mgL^{-1} and IAA 2.0 mgL^{-1} gave maximum number of multiple shoots when sucker was used as the explant for *in vitro* multiplication of banana cv. Grand Naine. (Ahmed *et al.*, 2014).

Male floral bud explants of banana cultivar 'Virupakshi' and 'Sirumalai' were cultured on MS basal medium supplemented with different combinations of growth supplements such as 6- benzylaminopurine (BAP), coconut water, naphthaleneacetic acid (NAA), gibberellic acid (Mahadev *et al.*, 2011). They observed that the media containing BAP 5.0 mgL^{-1} and coconut water 15 per cent as the most efficient media for initiation of shoot and showed highest per cent of culture establishment (94.4 per cent) and highest number of multiple shoots (15 shoots from a single part of a floral bud). Terminal male buds of Robusta (AAA) and Monthan (ABB) multiplied in MS media supplemented with BAP (5.0 mgL^{-1}) exhibited maximum number of shoots (4-12) (Balakrishnamurthy and Sree Rangaswamy, 1988).

Ganapathi *et al.* (1999) developed embryo cultures using male flowers in five banana cultivars namely 'Rasthali' (AAB), 'Basrai' (AAA), 'Shreemanti' (AAA), 'Lokandhi' (AAA) and 'Trikoni' (AAA). Embryonic callus rapidly proliferated on MS medium supplemented with 0.22 μM BA and 1.14 μM IAA. Among the five cultivars, Rasthali was found to be highly responsive (50 per cent) followed by Shreemanti (30 per cent) with minimum response found by Basrai, Lokandhi and Trikoni (8-15 per cent).

Regeneration of plants through indirect organogenesis in banana cultivar ' Sabri' using male flower buds showed greater response (20 per cent) in MS media supplemented with 2.0 mgL^{-1} 2,4-D, 0.5 mgL^{-1} NAA and 0.5 mgL^{-1} IAA and best multiplication of shoots (11 shoots regenerated from calli) were observed in media supplemented with 1.0 BA + 0.5 IAA + 500 CH (mgL^{-1}) (Sultan *et al.*, 2011).

Different cytokinins were found to induce varied effects in MS medium in which male flowers of Berangan (AAA), Rasthali (AAB), Nangka (AAB) and Abu (ABB) were cultured. Cultures became green after 10 to 15 days after their

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inoculation into the media supplemented with cytokinin and showed high proliferation, whereas, explants cultured onto the MS medium without any cytokinin did not show any proliferation. The highest number of 'CLBs' 4.5 and 3.9 were observed after 60 days in the media supplemented with 1.0 mgL^{-1} each of TDZ and BAP respectively. Optimum shoot regeneration was observed at 0.4, 0.6 and 0.8 mgL^{-1} of TDZ respectively, but all cultivars showed highest regeneration of shoots at 8.0 mgL^{-1} concentration of BAP (Darvari *et al.*, 2010).

Significant variation was observed in male bud culture using diploid cultivar Sannachenkadali (AA) and triploid cultivar Red banana (AAA) under different concentrations of BA and IAA (Resmi and Nair, 2007). Sannachenkadali (AA) recorded maximum number of multiple shoots in 8.9 μM 6-benzyl adenine (BA) whereas Red banana (AAA) recorded maximum multiplication in 22.2 μM 6-benzyl adenine. MS medium supplemented with 11.4 μM indole acetic acid and 17.8 μM BA were found suitable for shoot proliferation in triploid cultivar but not in the diploid cultivar.

Smitha *et al.*, (2014) studied the effect of TDZ on direct shoot regeneration from whole male inflorescence of four diploid banana cultivars from South India namely *Musa acuminata* cv. Matti, *M. acuminata* cv. Sannachenkadali, *M. acuminata* cv. Chingan and *Musa acuminata* cv.Njalipoovan (2n= 22) belonging to diploid genomic groups (AA and AB) and proved that TDZ was highly effective in producing more healthy shoots in *in vitro* propagation. The maximum number of shoots which were obtained in various concentration of TDZ ranging from 0.45 μM to 13.45 μM were not found to be influenced by genotypes. Other cytokinins such as BA and KIN were found to induce only lower number of regenerated shoots.

2.2.3. Vitamins

Murashige and Skoog (1962) medium consist of myo-inositol 100 mgL^{-1}, nicotinic acid 0.5 mgL^{-1} and pyridoxime 0.5 mgL^{-1} and when this is supplemented with glycine 0.2 mgL^{-1} and ascorbic acid 100 mgL^{-1} results were found to be

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favourable (Banerjee *et al.*, 1986). Thiamine is an essential vitamin often used at 0.4-1.0 mgL^{-1} in tissue culture (Krikorian and Cronauer, 1984).

2.3. Culture conditions

Tissue cultures are maintained in a culture room under controlled conditions of temperature, light and humidity and plays a significant role in the success of cultures. An optimum incubation temperature in the range of 26-30°C controlled by an air conditioning unit can satisfy the culture requirements of *Musa*. The temperature below 20°C and above 35°C has not been suggested in the culture room (Vuylsteke, 1989). Male bud cultures of banana maintained at a temperature of 25±2°C were recommended by several workers (Darvari *et al.*, 2010; Mahadev *et al.*, 2011; Sultan *et al.*, 2011).

Banana shoot tip cultures are usually kept under artificial lighting provided by cool, white fluorescent tubes. The success of proliferative growth and rooting of banana plantlets also depend on 12-16 h photoperiod (Banerjee *et al.*, 1986). At the multiplication stage of shoot tip cultures, 24 h light has to be provided. However, once shoots has rooted, they must be transferred to a 14/10 h light/dark cycle (Vuylsteke and De Langhe, 1985).

Maximum growth and development of axillary shoots in shoot tip culture were observed at a light intensity ranging from 80W to 120W for 16 h followed by 8 h dark period, while, in eye bud and floral apex culture, 80W light intensity was found to be the best (Bhaskar, 1991).

2.4. *In vitro* rooting

Spontaneous root formation rarely occur in some cultures under *in vitro* conditions. Moreover, shoots of majority of the species did not develop root system during multiplication stage of cultures. Rooting can be achieved either by subculturing to a medium lacking cytokinin, with or without a rooting hormone or by treating the shoots as conventional cuttings after removal from the sterile culture (Yeoman, 1986).

Auxins that are commonly used for root induction are NAA, IAA and IBA. High cytokinin levels are found to hinder the root induction. Hence investigators normally avoid the addition of cytokinin in the rooting stage. According to Gupta (1986) and Wong (1986) in certain cases, cytokinin can also promote rooting. Basal medium without any growth regulators can also be used for inducing rooting (Cronauer and Krikorian, 1984; Damasco and Barba, 1985; Jarret *et al.*, 1985; Sandoval, 1985).

Banerjee *et al.* (1986) found that NAA was more effective than IAA for rooting of *Musa* shoot tip cultures. In plantain cultivar Agbagba, more rooting (80 %) was observed in media supplemented with NAA at 1.0 μM and only 50% rooting was observed in media supplemented with IAA. Mante and Tepper (1983) and Cronauer and Krikorian (1984) found that the optimum range of NAA concentration for effective rooting is 0.2-1.0 mgL^{-1}.

IBA at 1.0 μM or even at 10-50 μM were also found to be effective in rooting of *Musa* cultivars (Doreswamy *et al.*, 1983; Mante and Tepper, 1983; Banerjee and De Langhe, 1985; Vuylsteke and De Langhe, 1985). MS medium supplemented with different concentration of IBA is best for good rooting of banana (Raut and Lokhande, 1989; Khanam *et al.*, 1996). Meanwhile, Bhaskar (1991) observed best rooting in banana in Knudson's medium supplemented with 5.0 mgL^{-1} NAA.

Rooting mainly occur in full strength of MS medium but rooting in medium with half concentration of macronutrients was suggested by Vuylsteke and De Langhe (1985) and Novak *et al.* (1990). Reduction of sucrose content from 3.0 % to 1-1.5% in rooting media was also suggested by Mante and Tepper (1983).

Cultures from male flowers were found to respond well to rooting hormones like IBA and NAA (Sultan *et al.*, 2011). Induction of rooting in *in vitro* culture of banana cultivar Sabri (AAB) was observed in 1.0 mgL^{-1} of IBA (5 roots per shoot) and NAA (4 roots per shoot).

Direct root regeneration in diploid banana cultivars Matti, Sannachenkadali, Njalipoovan and Chingan revealed that basal MS medium without any addition of supplement can result in good rooting within one week with no genotypic variation (Smitha *et al.*, 2014).

Mahadev *et al.*, (2011) found that per cent root induction was higher in male bud explants cultured on MS medium alone (81.7 per cent) than in combination with either IAA (1.0 or 1.5 mgL^{-1}) or charcoal (2.0 mgL^{-1}). Compared to other treatments, combination of MS with charcoal produced significantly more roots (76.7 per cent) than others. These findings were in confirmity with those of Resmi and Nair (2007) who used MS basal medium without any supplements and the cultures developed white, hairy roots within 10–15 days.

2.5. Hardening

In case of micropropagated plants, acclimatization is necessary because *in vitro* plant material is not adapted for *ex vitro* conditions (Brainerd and Funchigmi, 1981). The success of acclimatization of micropropagated plants depend upon pre-transfer culture conditions and post –transfer growth conditions (Ziv, 1986).

Plantlets having well-proportioned shoots and roots recorded more than 90 percentage survival rate during *in vitro* hardening. Once, roots are initiated, growth of shoot and development of roots were found to slow down in many of the plantain cultivars. Root ramification is observed when shoots and roots are continued to grow in the rooting medium leading to reduced survival percentage of the transplanted plantlets into the soil (Banerjee *et al.*, 1986).

According to George and Sherrington (1984) cooking bananas of ABB genotype developed sturdy plantlets with ramified roots when cultured in rooting medium for 4-6 weeks. Survival percentage of these plantlets exceeded 90% when transplanted to field. Application of triadimefon (1.0-2.0 mgL^{-1}) significantly improved the hardiness and survival ability of banana plantlets transferred from *in vitro* conditions to nursery (Murali and Duncan, 1995).

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2.5.1. Potting media

Perez and Hooks (2008) suggested a potting media which contains two parts of a commercial growing media mixture (Sunshine Professional), one part perlite, and three parts vermiculite (medium to coarse grade) to which a slow-release fertilizer is also added prior to planting for the best growth of the banana plantlets. Vasane and Kothari (2006) tried different types of potting mixture for planting *in vitro* propagated plantlets cv. Grand Naine. They observed that the plantlets performed well with all biometric parameters in the media containing press mud cake and poultry manure and the media containing coir pith resulted in good root growth.

Different potting media were used by Prabhuling (2010) for the successful hardening of tissue culture derived plantlets of banana cv. 'Grand Naine'. Coco pith was identified as the superior media while sand was not found suitable for primary hardening. Red soil+ coco pith+ sand (1:1:1 v/v) followed by red soil+ FYM+ sand (1:1:1 v/v) were found to be the best media for secondary hardening. Bhaskar (1991) reported the maximum survival percentage (90 per cent) of banana plantlets planted in the potting media containing vermiculite and sand (1:1 v/v).

According to Sapheera (2005) plantlets of cv. Njalipoovan and cv. Nivedyakadali survived better in the rooting medium containing a mixture of vermiculite and coir pith compost (1:1 v/v). Further growth of these plantlets were best in the potting mixture containing sand+ cow dung + goat manure (1:1:1).

In banana cv. 'Sabri', the regenerated plantlets from male flowers were established in small polybags containing garden soil and compost (1:1). The bags containing plantlets were sprayed with water at 24 h interval to maintain humidity around the plantlets and to get acclimatized to outdoor conditions. Out of this, 90 per cent plantlets got established successfully in the field (Sultan *et al.*, 2011). When, rooted shoots of Virupakshi and Sirumalai were transferred to soil mixture containing sterile sand, soil and humus at the ratio of 1:2:1 for hardening, per cent survival was noticed (Mahadev *et al.*, 2011).

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Somatic embryo derived plantlets with good root system survived successfully and normal plant development were noticed when these plantlets were grown inside the greenhouse in paper cups containing soilrite (Ganapathi *et al.*, 1999). According to Smitha *et al.*, (2014), when plantlets after attaining 5 cm height were transferred to vermiculite media under culture room conditions for three weeks and then transferred and maintained under greenhouse condition for three to four weeks and finally transplanted to field resulted in 80 per cent survival.

Resmi and Nair (2007) evaluated the survival rate of plantlets of *Musa acuminata* in South India. Rooted plantlets were first transferred to plastic cups filled with vermiculite, then covered with transparent plastic bags and maintained at a temperature of 25 ± 2°C for 10 days and then again at room temperature (30–32°C) for 2 weeks before planting in the field resulted in 100 per cent survival.

Materials and Methods

3. MATERIALS AND METHODS

The studies on *in vitro* propagation of banana *Musa* (AA) 'Kadali' were carried out in the Plant Tissue Culture Laboratory at Banana Research Station, Kannara during 2016-2018. The details regarding the experimental materials (Table 1), methodology of experiments (Table 2) and analytical techniques adopted are presented stepwise in this chapter.

Table 1. Experimental Materials

Crop	Banana Kadali (AA)
Explant used	20-25 days old male bud
Media for establishment	Half and Full MS media with 4 levels of NAA and 4 levels of BA
Media for multiple shoot induction	Half and Full MS media with 2 levels of NAA and 5 levels of BA
Media for root induction and acclimatization	Half and Full MS media with 2 levels of Sucrose and 4 levels of IBA

Table 2. Experimental Methods

Media standardization for establishment	
Treatments	32
Media	2
PGR levels	4x4
Design	CRD
Replications	3
No of cultures/treatment	6
Media standardization for multiple shoot induction	
Treatments	20
Media	2
PGR levels	2x5

Design	CRD
Replications	3
No of cultures/treatment	6
Media standardization for root induction and acclimatization	
Treatments	16
Media	2
Sucrose levels	2
IBA levels	4
Design	CRD
Replications	3
No of cultures/treatment	6

3.1. Chemicals

The major and minor elements used for the preparation of media were of analytical grade and procured from British Drug House and Sisco Research Laboratory, and plant growth regulators were obtained from Sigma, USA.

3.2. Glassware

Borosilicate glassware of borosil brand was used for the study. They were cleaned by initial soaking in potassium dichromate solution in sulphuric acid for 12 hours followed by thorough washing with jets of tap water in order to remove all traces of dichromate solution. They were then soaked in detergent solution (cleansol) 0.1 per cent overnight, then thoroughly washed with tap water and rinsed twice with double distilled water. The glasswares were then dried in a hot air oven at 100° C for 24 hours and stored in cupboards away from dust and contaminants until used.

3.3. Culture medium

3.3.1. Medium composition

The most commonly used MS medium (Murashige and Skoog, 1962) with certain modifications was used in the present study. The nutrient medium included inorganic salts, vitamins, growth regulators, organic supplements and a carbon source. The composition of the basal medium is given as Appendix.

3.3.2. Preparation of the medium

Media was prepared by following standard procedures (Gamborg and Shyluk, 1981). Initially, stock solutions of major and minor nutrients, vitamins, Fe-EDTA and growth regulators were prepared by dissolving the required quantities of chemicals in double distilled water and stored under refrigerated conditions in amber coloured bottle. The stock solutions of vitamins, amino acids and phytohormones were prepared fresh every week and that of nutrients were prepared fresh every four weeks. Required volume of the stock solutions of chemicals and phytohormones were pipetted out into a 1000 ml beaker. Sucrose and inositol were freshly added and dissolved by stirring. Finally, the volume was made upto 1000 ml by adding double distilled water. Agar was added as solidifying agent at 0.7 per cent (w/v) concentration after adjusting the pH of the solution using pH paper by adding 0.1 N NaOH or 0.1 N HCl. The medium was stirred and heated to melt the agar by keeping the solution in a water bath maintained at 90°C to 95°C. The medium (250 ml) was poured when hot into the oven sterilized culture vessels and were plugged tightly with non-absorbent cotton. Vessels containing the media were autoclaved at 121°C and 15 psi pressure (1.06 kg cm^{-2}) for twenty minutes. After sterilization, the medium were allowed to cool to room temperature and stored in the culture room until used.

3.4. Transfer area and aseptic manipulations

All the aseptic manipulations such as surface sterilization of explants, preparation and inoculation of explants and subsequent subculturing were carried out under the hood of a clean ' Thermadyne' laminar airflow cabinet. The working

table of the laminar air flow cabinet was first sterilized by wiping with absolute alcohol and then by putting on the UV light for 30 minutes. The bottles as well as the instruments used for the inoculation were first steam sterilized in an autoclave at 15 psi at 121°C for 25 minutes and then flame sterilized before each inoculation. Hands were wiped with alcohol before each inoculation.

3.5. Culture room

The cultures were incubated at 25±2° C in an air conditioned culture room with a 16 hour of photoperiod and 2000 lux light intensity supplied by cool white fluorescent tubes.

3.6. Selection of the variety

The explants for the study were collected from a popular diploid banana cultivar of Kerala, *Musa* (AA) ' Kadali'.

3.7. Source of explant

Male buds of *Musa* (AA) 'Kadali' were collected from farmers fields in Thrissur district. Male flower buds from field grown banana plants were collected at 20-25 days after the emergence of bunch and used for the present study.

3.8. Collection and preparation of explants for culturing

Healthy male flower buds of 20-25 days old after bunch emergence were collected from the field (Plate 1). The bracts covering the hands of male buds were removed in a stepwise manner upto 35-45 according to the size of male bud without making any injury until it reached a length of 4-5 cm under non-sterile conditions (Plate 2). The male buds were then washed four times thoroughly in tap water. Then these were again washed with antibacterial soap solution and aquaguard water. Further sterilization procedures were carried out under perfect aseptic conditions in a 'Thermadyne' laminar airflow cabinet.

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Plate 1. Male bud of Musa (AA) Kadali

Plate 2. Male bud after the removal of bracts

Plate 3. Explants from one male bud

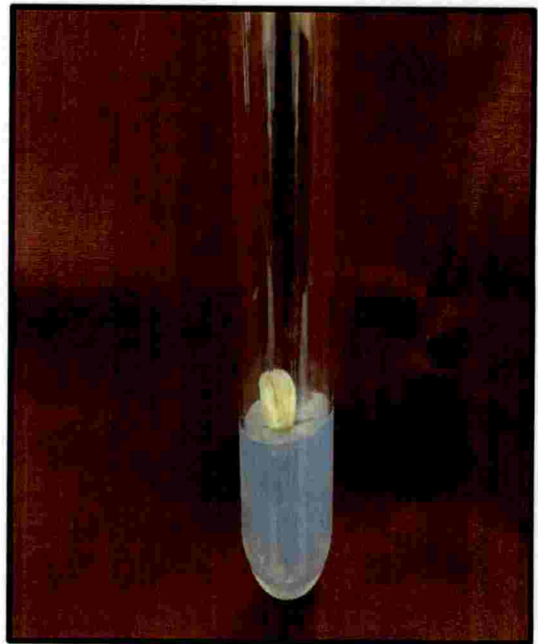

Plate 4. Inoculated explant in culture tube

3.9. Inoculation of explants

The explants were surface sterilized with 0.1 per cent (w/v) mercuric chloride for for minutes followed by three rinses in autoclaved double distilled water in the laminar air flow cabinet. Protective bracts about 45-50 numbers covering the male flowers were removed by sterilized blade and forceps and male flowers having size 0.5-1.0 cm were taken out and placed in sterilized plates (Plate 3). Each male bud contained approximately 10-15 explants were taken out and inoculated in suitable culture medium (Plate 4).

3.10. Explant establishment

3.10.1. Standardisation of establishment media

The most commonly used MS medium (Murashige and Skoog, 1962) was tried for male bud explants to study the morphogenic response of diploid banana cultivar Kadali. The basal media was modified with the following treatments.

Media	Combinations
Half MS and Full MS	4 levels of BA (1.0, 2.0, 3.0, 4.0 mgL^{-1}) and 4 levels of NAA (0, 0.5, 1.0 and 1.5 mgL^{-1})

Observations on the number of days taken for culture establishment, percentage of cultures established and contamination percentage were made after four weeks of culturing.

3.11. Induction of axillary shoots and rapid shoot multiplication

3.11.1. Standardization of multiple shoot induction media

The explants were transferred to fresh media at three weeks interval until the formation of shoot buds. Axillary shoots of 1.0-1.5 cm length from established culture were used for shoot multiplication.

To study the effect on multiplication of shoots in cultures and to identify the best media for multiplication, both full MS and half MS media supplemented with

various concentration of growth regulators were used. The details of the trial using growth regulators *viz.*, cytokinins and auxins are given below.

Media	Combinations
Half MS and Full MS	2 levels of NAA (0.5 and 1.0 mgL^{-1}) and 5 levels of BA (2.0, 4.0, 6.0, 8.0 and 10.0 mgL^{-1})

The best two media identified from the above trial were modified by adding the media supplements, 5 levels of TDZ (0.2, 0.4, 0.6, 0.8 and 1.0 mgL^{-1}) and 3 levels of coconut water (5,10 and 15%). There were six cultures per treatment. The multiple shoots induced were separated into clumps of two to three shoots clump and subcultured at 30 days interval. Observations on per cent of cultures developing shoots, number of shoots per culture, length of micro shoot after one month (cm), number of leaves and number of roots were recorded after 90 days of subculturing.

3.12. *In vitro* rooting of banana

3.12.1. Standardization of *in vitro* rooting

The study on *in vitro* rooting was conducted on half and full MS media. Shoots (2.0 - 3.0 cm in length) excised from proliferated shoot cultures were transferred to the root induction media. The media supplemented with different levels of carbon source and auxin for rooting of Kadali are given below

Media	Combinations
Half MS and Full MS	2 levels of sucrose (1.5 and 3.0 %) and 4 levels of IBA (1.0, 2.0, 3.0 and 4.0 mgL^{-1})

Observations for number of days taken for root initiation, number of roots, per cent of rooted plants and length of longest root (cm) were recorded on six cultures per treatment after four weeks of culturing. The *in vitro* cultures were

incubated under 25±2°C in an air conditioned culture room with 16 hour photoperiod and 2000 lux light intensity.

3.13. Acclimatization

The rooted plantlets from the rooting media were taken out from the jam bottles using foreceps, after soaking the media in water for five minutes. The plantlets were then washed in running water to remove the adhering solid medium. Then the plantlets were dipped in bavistin solution (0.3 %) and again washed in water. The well rooted plantlets were then planted in the pro trays containing 1:1 (v/v) of sterilized cocopeat and vermiculite and kept in green house. After one month of hardening, the acclimatized plants were transferred into the potting media containing 1:1:1 (v/v) mixture of sand + cowdung + goat manure.

The plants were sprayed with nutrient solution at weekly intervals. The morphological observations were recorded at 30 days after planting. Observations on per cent of plantlet survival and other morphological traits of thirty plants were recorded. The major morphological characters for which observations were taken include:

3.13.1. Vegetative characters

1. Plant height (cm)

   Recorded from the base of pseudostem to the base of the unopened leaf.

2. Number of leaves

   All green leaves persisting except the young folded one were recorded.

3. Leaf length and breadth (cm)

   Recorded from the fully unfolded third leaf counting down from the top of the plant.

3.14. Statistical analysis

Data were statistically analysed by applying analysis of variance (ANOVA) as per the design adopted in the experiment with the help of online statistical package ' OP stat' (Sheoran *et al.*, 1998)

Results

4. RESULTS

The results of the various experiments conducted to standardise the *in vitro* male bud culture in banana *Musa* (AA) 'Kadali' at the Plant Tissue Culture Laboratory, Banana Research Station, Kannara are presented in the following sections.

4.1. Standardization of establishment media

Successful proliferation of cultures of male buds were observed in all the media combinations tried under this experiment. All the cultures were found to be free from contamination.

4.1.1. Number of days taken for culture establishment

Data regarding the effect of MS media, NAA and BA at different concentrations on the number of days taken for the culture establishment is given in Table 3. It was observed that the MS media significantly influenced the number of days taken for culture establishment. The male bud cultures took least number of days (29.24) for culture establishment in full MS medium. The medium supplemented with NAA and BA at different concentrations had significant influence on number of days taken for establishment of cultures. Similarly, when the effect of NAA in general was viewed NAA 1.5 mgL^{-1} was judged as the best (29.43), which was on par with lower concentrations of NAA at 0.5 mgL^{-1} (29.54) and 1.0 mgL^{-1} (29.52). When the effect of BA alone was considered, BA at 4.0 mgL^{-1} recorded the least number of days for culture establishment (28.64) and was significantly superior to all the other treatments.

The differential response of MS media supplemented with different concentrations of NAA and BA is presented in Table 3a. Among the combinations, the earliest culture establishment (26.99) was noted in full MS medium containing NAA 1.0 mgL^{-1} and BA 4.0 mgL^{-1} (Plate 5) and was significantly superior to all the other treatments.

Plate 5. Culture establishment of male bud explants in full MS + NAA 1.0 mgL^{-1} + BA 4.0 mgL^{-1}

4.1.2. Percentage of cultures established

The effect of various treatments on percentage of cultures established are given in Table 3.

With regard to the percentage of established cultures, differential response was noticed with respect to the addition of BA. BA 4.0 mgL^{-1} was found to be significantly superior to all other treatments and recorded highest percentage of established cultures (93.05).

Considering the percentage of cultures established, a differential response was viewed under full MS and half MS media supplemented with different levels of BA (Table 3b). Highest percentage of cultures established (97.22) was observed under full MS medium supplemented with BA 4.0 mgL^{-1} which was on par with BA 4.0 mgL^{-1} in half MS medium (88.89).

4.2. Standardization of multiple shoot induction media

4.2.1. Cultures developing shoots

Data pertaining to the effect of MS media, NAA and BA on cultures developing shoots are given in Table 4.

Concerning this character, significant response was noticed only with respect to different concentration of BA. Cent per cent of the cultures containing BA 4.0 mgL^{-1} and 6.0 mgL^{-1} developed shoots which remained statistically on par with BA 8.0 mgL^{-1} (98.61). On the contrary, the interaction effect among the combinations of MS media, NAA and BA were not found to significantly influence the development of shoots in cultures.

4.2.2. Number of shoots per culture

The data presented in Table 4 indicated the effect of MS media, NAA and BA on number of shoots per culture. The data revealed that all the treatments had significant influence on number of shoots per culture. The influence of MS media showed that, cultures in full MS resulted in maximum number of shoots per culture

Table 3. Influence of MS media, NAA (mgL^{-1}) and BA (mgL^{-1}) levels on number of days taken for culture establishment and percentage of cultures established.

Treatments	Number of days taken for culture establishment	Percentage of cultures established
Media		
Half MS	29.80	71.18
Full MS	29.24	70.14
CD (0.05)	0.10	NS
NAA(mgL^{-1})		
NAA 0.0	29.66	70.83
NAA 0.5	29.54	70.83
NAA 1.0	29.52	75.69
NAA 1.5	29.43	65.27
CD (0.05)	0.15	NS
BA (mgL^{-1})		
BA 1.0	30.33	36.11
BA 2.0	29.66	73.61
BA 3.0	29.52	79.86
BA 4.0	28.64	93.05
CD (0.05)	0.15	7.66

Table 3a. Interaction effect of MS, NAA (mgL^{-1}) and BA (mgL^{-1}) on number of days taken for culture establishment.

Treatments	Number of days taken for culture establishment
Half MS + NAA0.0 + BA1.0	30.33
Half MS + NAA0.0 + BA2.0	29.66
Half MS + NAA0.0 + BA3.0	29.66
Half MS + NAA0.0 + BA4.0	29.33
Half MS + NAA0.5 + BA1.0	30.66
Half MS + NAA 0.5+ BA2.0	29.66
Half MS + NAA 0.5+ BA3.0	29.83
Half MS + NAA 0.5+ BA4.0	29.50
Half MS + NAA1.0 + BA1.0	30.66
Half MS + NAA1.0 + BA2.0	30.00
Half MS + NAA1.0 + BA3.0	29.83
Half MS + NAA1.0 + BA4.0	29.33
Half MS + NAA1.5 + BA1.0	30.16
Half MS + NAA1.5 + BA2.0	29.49
Half MS + NAA1.5 + BA3.0	29.83
Half MS + NAA1.5 + BA4.0	29.49
Full MS + NAA0.0 + BA1.0	30.33
Full MS + NAA0.0 + BA2.0	29.33
Full MS + NAA0.0 + BA3.0	29.49
Full MS + NAA0.0 + BA4.0	29.16
Full MS + NAA0.5 + BA1.0	30.33
Full MS + NAA 0.5+ BA2.0	29.83
Full MS + NAA 0.5+ BA3.0	28.83
Full MS + NAA 0.5+ BA4.0	27.66
Full MS + NAA1.0 + BA1.0	30.16
Full MS + NAA1.0 + BA2.0	29.83
Full MS + NAA1.0 + BA3.0	29.33
Full MS + NAA1.0 + BA4.0	26.99
Full MS + NAA1.5 + BA1.0	29.99
Full MS + NAA1.5 + BA2.0	29.49
Full MS + NAA1.5 + BA3.0	29.33
Full MS + NAA1.5 + BA4.0	27.66
CD (0.05)	0.41

Table 3b. Interaction effect of MS media and BA (mgL^{-1}) on percentage of cultures established

BA(mgL^{-1})	Percentage of cultures established	
	Half MS	Full MS
BA 1.0	44.44	27.77
BA 2.0	76.39	70.83
BA 3.0	74.99	84.72
BA 4.0	88.89	97.22
CD (0.05)	10.84	

Table 4. Influence of MS media, NAA (mgL^{-1}) and BA (mgL^{-1}) levels on cultures developing shoots and number of shoots per culture.

Treatments	Cultures developing shoots	Number of shoots per culture
Media		
Half MS	96.67	10.36
Full MS	96.11	11.85
CD (0.05)	NS	0.25
NAA(mgL^{-1})		
NAA 0.5	97.22	11.40
NAA 1.0	95.56	10.81
CD (0.05)	NS	0.25
BA (mgL^{-1})		
BA 2.0	94.44	9.83
BA 4.0	100.00	12.08
BA 6.0	100.00	12.62
BA 8.0	98.61	11.20
BA 10.0	88.89	9.79
CD (0.05)	5.02	0.39

(11.85) and found superior to half MS media (10.36). When considering the levels of NAA, NAA 0.5 mgL^{-1} recorded more number of shoots per culture (11.40) and was superior to NAA 1.0 mgL^{-1}(10.81). With regard to the different concentration of BA, BA at 6.0 mgL^{-1} showed maximum number of shoots per culture (12.62) and was superior to all other treatments.

Data presented in Table 4a indicated the significant response of MS media containing different levels of NAA and BA with respect to number of shoots per culture. Maximum number of shoots per culture (15.99) were observed in full MS medium supplemented with NAA 1.0 mgL^{-1} and BA 4.0 mgL^{-1} (Plate 6) and was superior to all other treatment combinations. The lowest (8.66) number of shoots per culture was recorded in half MS media supplemented with NAA 0.5 mgL^{-1} and BA 2.0 mgL^{-1}.

4.2.3. Length of micro shoot after one month

The data pertaining to the effect of MS media, NAA and BA at different levels on length of micro shoot after one month of subculturing is furnished in Table 4b. MS media significantly influenced the micro shoot length which was significantly higher (0.57cm) in full MS media. With respect to NAA, NAA 1.0 mgL^{-1} recorded the tallest micro shoot after one month (0.57cm). Among different levels of BA tried in multiple shoot induction media, tallest micro shoot (0.58 cm) was recorded in BA 4.0 mgL^{-1} which was on par with BA 6.0 mgL^{-1} (0.57cm).

Interaction effect of MS media with NAA and BA were found to significantly effect the length of micro shoots (Table 4c). Among the different treatment combinations, NAA 1.0 mgL^{-1} in combination with BA 6.0 mgL^{-1} in full MS recorded highest value for micro shoot after one month (0.91cm) which was on par with full MS media supplemented with NAA 1.0 mgL^{-1} and BA 4.0 mgL^{-1} (0.86 cm).

4.2.4. Number of leaves

Data regarding the effect of various treatments on number of leaves are given in Table 4b.

Among the media, full MS was noticed as the best in significantly influencing the number of leaves. Maximum number of leaves (14.25) were noticed in full MS media and was significantly superior to half MS media (11.34). Cultures supplemented with NAA 0.5 mgL^{-1} was found to be superior in inducing more number of leaves (13.44). When the effect of BA alone was considered, BA at different concentrations significantly affected the number of leaves. Maximum number of leaves (14.96) were noticed under 6.0 mgL^{-1} BA and was closely followed by 4.0 mgL^{-1} BA (14.14).

Data presented in Table 4c revealed the interaction effect of MS media supplemented with different concentrations of NAA and BA on the number of leaves. When the interaction effect of combinations of full MS supplemented with NAA and BA were studied, NAA 1.0 mgL^{-1} and BA 6.0 mgL^{-1} recorded maximum number of leaves (19.83) and was on par with full MS media supplied with NAA 1.0 mgL^{-1} and BA 4.0 mgL^{-1} (19.50). Lowest number of leaves (8.0) were noticed in half MS media supplemented with NAA 1.0 mgL^{-1} and BA 8.0 mgL^{-1} (Table 5a).

4.2.5. Number of roots

The emergence of roots was noticed at the multiple shoot induction stage itself which is not desirable for the multiplication stage as at this stage we prefer more number of shoots. However, irrespective of the treatments, root initiation was noticed with no significant difference (Table 4b).

On the contrary, interaction effect of MS media, NAA and BA were found to significantly influence the number of roots in cultures (Table 4c). Maximum number of roots (18.07) were observed in half MS medium containing NAA 1.0 mgL^{-1} and BA 2.0 mgL^{-1} which was on par with half MS media supplemented with NAA 0.5 mgL^{-1} + BA 2.0 mgL^{-1} (15.11) and NAA 0.5 mgL^{-1} + BA 4.0 mgL^{-1}(15.11).

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Table 4a. Interaction effect of MS media, NAA (mgL^{-1}) and BA (mgL^{-1}) on number of shoots per culture.

Treatments	Number of shoots per culture
Half MS + NAA0.5 + BA2.0	8.66
Half MS + NAA0.5 + BA4.0	10.49
Half MS + NAA 0.5+ BA6.0	11.66
Half MS + NAA 0.5+ BA8.0	13.33
Half MS + NAA 0.5+ BA10.0	10.16
Half MS + NAA1.0 + BA2.0	9.99
Half MS + NAA1.0 + BA4.0	9.99
Half MS + NAA1.0 + BA6.0	10.49
Half MS + NAA1.0 + BA8.0	9.49
Half MS + NAA1.0 + BA10.0	9.33
Full MS + NAA0.5 + BA2.0	11.00
Full MS + NAA0.5 + BA4.0	11.83
Full MS + NAA 0.5+ BA6.0	13.66
Full MS + NAA 0.5+ BA8.0	12.83
Full MS + NAA 0.5+ BA10.0	10.33
Full MS + NAA1.0 + BA2.0	9.66
Full MS + NAA1.0 + BA4.0	15.99
Full MS + NAA1.0 + BA6.0	14.66
Full MS + NAA1.0 + BA8.0	9.16
Full MS + NAA1.0 + BA10.0	9.33
CD (0.05)	0.78

Table 4b. Influence of MS media, NAA (mgL^{-1}) and BA (mgL^{-1}) on length of micro shoot after one month, number of leaves and number of roots.

Treatments	Length of micro shoot after 1 month (cm)	Number of leaves	Number of roots
Media			
Half MS	0.42	11.34	13.06
Full MS	0.57	14.25	11.20
CD (0.05)	0.04	0.70	NS
NAA(mgL^{-1})			
NAA 0.5	0.42	13.44	12.28
NAA 1.0	0.57	12.15	11.99
CD (0.05)	0.04	0.70	NS
BA (mgL^{-1})			
BA 2.0	0.41	12.71	13.10
BA 4.0	0.58	14.14	13.58
BA 6.0	0.57	14.96	12.36
BA 8.0	0.49	12.37	10.51
BA 10.0	0.43	9.79	11.12
CD (0.05)	0.06	1.11	NS

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Table 4c. Interaction effect of MS media, NAA (mgL^{-1}) and BA (mgL^{-1}) on length of micro shoot after one month, number of leaves and number of roots.

Treatments	Length of micro shoot after one month (cm)	Number of leaves	Number of roots
Half MS + NAA0.5 + BA2.0	0.39	13.00	15.11
Half MS + NAA0.5 + BA4.0	0.41	11.58	15.11
Half MS + NAA0.5 + BA6.0	0.49	12.17	14.44
Half MS + NAA0.5 + BA8.0	0.38	14.33	11.44
Half MS + NAA0.5 + BA10.0	0.30	12.66	13.44
Half MS + NAA1.0 + BA2.0	0.26	11.33	18.07
Half MS + NAA1.0 + BA4.0	0.56	9.66	13.66
Half MS + NAA1.0 + BA6.0	0.39	12.50	13.55
Half MS + NAA1.0 + BA8.0	0.48	8.00	7.77
Half MS + NAA1.0 + BA10.0	0.49	8.16	8.05
Full MS + NAA0.5 + BA2.0	0.41	14.83	14.00
Full MS + NAA0.5 + BA4.0	0.48	15.83	11.33
Full MS + NAA 0.5+ BA6.0	0.50	15.33	9.89
Full MS + NAA 0.5+ BA8.0	0.43	14.66	9.83
Full MS + NAA 0.5+ BA10.0	0.43	10.00	8.22
Full MS + NAA1.0 + BA2.0	0.55	11.66	5.22
Full MS + NAA1.0 + BA4.0	0.86	19.50	14.22
Full MS + NAA1.0 + BA6.0	0.91	19.83	11.55
Full MS + NAA1.0 + BA8.0	0.65	12.50	12.99
Full MS + NAA1.0 + BA10.0	0.51	8.33	14.77
CD(0.05)	0.13	2.22	6.77

4.3. Effect of media supplements

From the multiple shoot induction, two best media (combinations of NAA 1.0 mgL^{-1} with BA 4.0 mgL^{-1} and 6.0 mgL^{-1} under full MS) were identified and these two media were then modified by adding different levels of TDZ and coconut water. Under this trial, cent per cent of cultures were developed into shoots.

4.3.1. Number of shoots per culture

The data on influence of different treatments on number of shoots per culture is given in Table 5. Maximum number of shoots per culture (15.90) was recorded in BA 4.0 mgL^{-1} which was found to be superior to BA 6.0 mgL^{-1} (15.66). Among the various levels of coconut water used, media containing 15 per cent of coconut water recorded the maximum number of shoots (16.97), which remained statistically superior to the other two treatments. Number of shoots varied significantly among TDZ levels. At different concentration of TDZ tried, maximum number of shoots (17.12) were noted in 0.4 mgL^{-1} TDZ which was superior to all other treatments.

A differential response was observed in combinations of BA, coconut water and TDZ on the production of number of shoots per culture (Table 5a). Maximum value (19.38) was observed in the treatment combination BA 4.0 mgL^{-1} along with coconut water 15 per cent and TDZ 0.4 mgL^{-1} (Plate 7) which was on par with combination of BA 6.0 mgL^{-1} with same amount of coconut water and TDZ (18.99).

4.3.2. Length of micro shoot after one month

Length of micro shoot was uniform and was least influenced by the combinations. Statistically, the values were found to be non significant.

Plate 6. ***In vitro*** **multiple shoot induction in full MS + NAA 1.0 mgL^{-1} + BA 4.0 mgL^{-1}**

Plate 7. ***In vitro*** **multiple shoot induction in modified media supplemented with BA 4.0 mgL^{-1} + CW 15 % + TDZ 0.4 mgL^{-1}**

4.3.3. Number of leaves

The data pertaining to the influence of BA, coconut water and TDZ levels on number of leaves in cultures are given in Table 5.

With regard to BA, significant influence was noticed on the number of leaves in the multiple shoot induction cultures. Higher number of leaves (18.53) were observed at BA 4.0 mgL^{-1}. Different levels of coconut water were also found to have significant influence on number of leaves. Higher number of leaves (19.17) were recorded at 15 per cent coconut water and found superior to other two levels. TDZ level at 0.4 mgL^{-1} recorded higher number of leaves (19.40) which was found to be significantly superior to all the other levels.

Differential response was noticed in combinations of BA with coconut water and TDZ on multiple shoot formation (Table 5b). Among the different additives, full MS media supplemented with 6.0 mgL^{-1} BA, 15 per cent coconut water and TDZ 0.4 mgL^{-1} recorded highest number of leaves (22.66) and was on par with the combination BA 4.0 mgL^{-1}, coconut water 15 per cent and 0.2 mgL^{-1} TDZ (22.16).

4.3.4. Number of roots

In terms of number of roots, no significant difference was noticed (Table 5). Though, rooting in shooting media is undesirable, rooting was observed in all the treatment combinations.

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Table 5. Influence of BA (mgL^{-1}), CW (%) and TDZ (mgL^{-1}) on number of shoots per culture, length of micro shoot after one month, number of leaves and number of roots.

Treatments	Number of shoots per culture	Length of micro shoot after 1 month (cm)	Number of leaves	Number of roots
BA(mgL^{-1})				
BA 4.0	15.90	0.95	18.53	10.62
BA 6.0	15.66	0.91	17.32	11.60
CD (0.05)	0.19	NS	0.16	NS
CW (%)				
CW 5%	15.37	0.89	17.14	10.93
CW 10%	15.00	0.92	17.47	11.65
CW 15%	16.97	0.98	19.17	10.76
CD (0.05)	0.23	NS	0.20	NS
TDZ(mgL^{-1})				
TDZ 0.2	14.63	0.91	18.21	11.03
TDZ 0.4	17.12	0.93	19.40	11.63
TDZ 0.6	16.41	0.95	18.50	11.57
TDZ 0.8	16.13	0.94	18.11	10.15
TDZ 1.0	14.62	0.93	15.41	11.20
CD (0.05)	0.29	NS	0.26	NS

Table 5a. Interaction effect of BA (mgL^{-1}) CW (%) and TDZ (mgL^{-1}) on number of shoots per culture.

Treatments	Number of shoots per culture
BA 4.0 + CW 5.00 + TDZ 0.2	15.66
BA 4.0 + CW 5.00 + TDZ 0.4	14.89
BA 4.0 + CW 5.00 + TDZ 0.6	15.66
BA 4.0 + CW 5.00 + TDZ 0.8	15.49
BA 4.0 + CW 5.00 + TDZ 1.0	15.00
BA 4.0 + CW 10.0 + TDZ 0.2	14.00
BA 4.0 + CW 10.0 + TDZ 0.4	17.39
BA 4.0 + CW 10.0 + TDZ 0.6	16.49
BA 4.0 + CW 10.0 + TDZ 0.8	15.44
BA 4.0 + CW 10.0 + TDZ 1.0	14.74
BA 4.0 + CW 15.0 + TDZ 0.2	14.78
BA 4.0 + CW 15.0 + TDZ 0.4	19.38
BA 4.0 + CW 15.0 + TDZ 0.6	18.22
BA 4.0 + CW 15.0 + TDZ 0.8	17.33
BA 4.0 + CW 15.0 + TDZ 1.0	14.05
BA 6.0 + CW 5.00 + TDZ 0.2	15.16
BA 6.0 + CW 5.00 + TDZ 0.4	16.39
BA 6.0 + CW 5.00 + TDZ 0.6	15.78
BA 6.0 + CW 5.00 + TDZ 0.8	16.16
BA 6.0 + CW 5.00 + TDZ 1.0	13.50
BA 6.0 + CW 10.0 + TDZ 0.2	13.00
BA 6.0 + CW 10.0 + TDZ 0.4	15.66
BA 6.0 + CW 10.0 + TDZ 0.6	14.66
BA 6.0 + CW 10.0 + TDZ 0.8	15.33
BA 6.0 + CW 10.0 + TDZ 1.0	13.25
BA 6.0 + CW 15.0 + TDZ 0.2	15.17
BA 6.0 + CW 15.0 + TDZ 0.4	18.99
BA 6.0 + CW 15.0 + TDZ 0.6	17.66
BA 6.0 + CW 15.0 + TDZ 0.8	16.99
BA 6.0 + CW 15.0 + TDZ 1.0	17.16
CD (0.05)	0.72

Table 5b. Interaction effect of BA (mgL^{-1}), CW(%) and TDZ (mgL^{-1}) on number of leaves.

Treatments	Number of leaves
BA 4.0 + CW 5.00 + TDZ 0.2	20.16
BA 4.0 + CW 5.00 + TDZ 0.4	19.50
BA 4.0 + CW 5.00 + TDZ 0.6	16.00
BA 4.0 + CW 5.00 + TDZ 0.8	18.16
BA 4.0 + CW 5.00 + TDZ 1.0	16.66
BA 4.0 + CW 10.0 + TDZ 0.2	16.00
BA 4.0 + CW 10.0 + TDZ 0.4	20.16
BA 4.0 + CW 10.0 + TDZ 0.6	19.49
BA 4.0 + CW 10.0 + TDZ 0.8	18.16
BA 4.0 + CW 10.0 + TDZ 1.0	17.33
BA 4.0 + CW 15.0 + TDZ 0.2	22.16
BA 4.0 + CW 15.0 + TDZ 0.4	21.00
BA 4.0 + CW 15.0 + TDZ 0.6	20.33
BA 4.0 + CW 15.0 + TDZ 0.8	18.66
BA 4.0 + CW 15.0 + TDZ 1.0	14.11
BA 6.0 + CW 5.00 + TDZ 0.2	16.00
BA 6.0 + CW 5.00 + TDZ 0.4	16.00
BA 6.0 + CW 5.00 + TDZ 0.6	17.25
BA 6.0 + CW 5.00 + TDZ 0.8	17.99
BA 6.0 + CW 5.00 + TDZ 1.0	13.66
BA 6.0 + CW 10.0 + TDZ 0.2	17.44
BA 6.0 + CW 10.0 + TDZ 0.4	17.05
BA 6.0 + CW 10.0 + TDZ 0.6	17.33
BA 6.0 + CW 10.0 + TDZ 0.8	16.33
BA 6.0 + CW 10.0 + TDZ 1.0	15.38
BA 6.0 + CW 15.0 + TDZ 0.2	17.50
BA 6.0 + CW 15.0 + TDZ 0.4	22.66
BA 6.0 + CW 15.0 + TDZ 0.6	20.57
BA 6.0 + CW 15.0 + TDZ 0.8	19.33
BA 6.0 + CW 15.0 + TDZ 1.0	15.33
CD (0.05)	0.64

Table 6. Influence of MS media, sucrose (%) and IBA (mgL^{-1}) on number of days taken for root initiation, number of roots and length of longest root.

Treatments	Number of days taken for root initiation	Number of roots	Length of longest root (cm)
Media			
Half MS	6.29	3.75	6.13
Full MS	6.33	4.23	4.06
CD (0.05)	NS	0.38	0.52
Sucrose (%)			
Sucrose 1.5	6.37	3.14	4.96
Sucrose 3.0	6.25	4.83	5.24
CD (0.05)	NS	0.38	NS
IBA (mgL^{-1})			
IBA 1.0	5.08	4.54	6.41
IBA 2.0	6.45	4.08	4.95
IBA 3.0	6.70	3.29	5.79
IBA 4.0	7.00	4.04	3.23
CD (0.05)	0.34	0.54	0.74

Table 6a. Interaction effect of MS media, sucrose (%) and IBA (mgL^{-1}) on rooted plants (%) and number of days taken for root initiation.

Treatments	Rooted plants (%)	Number of days taken for root initiation
Half MS+ sucrose 1.5 + IBA 1.0	100	5.33
Half MS+ sucrose 1.5 + IBA 2.0	100	5.66
Half MS+ sucrose 1.5 + IBA 3.0	100	6.49
Half MS+ sucrose 1.5 + IBA 4.0	100	7.33
Half MS+ sucrose 3.0 + IBA 1.0	100	5.16
Half MS+ sucrose 3.0 + IBA 2.0	100	6.99
Half MS+ sucrose 3.0 + IBA 3.0	100	6.66
Half MS+ sucrose 4.0 + IBA 4.0	100	6.6
Full MS+ sucrose 1.5 + IBA 1.0	100	5.16
Full MS+ sucrose 1.5 + IBA 2.0	100	6.99
Full MS+ sucrose 1.5 + IBA 3.0	100	6.99
Full MS+ sucrose 1.5 + IBA 4.0	100	6.99
Full MS+ sucrose 3.0 + IBA 1.0	100	4.66
Full MS+ sucrose 3.0 + IBA 2.0	100	6.16
Full MS+ sucrose 3.0 + IBA 3.0	100	6.66
Full MS+ sucrose 4.0 + IBA 4.0	100	7.00
CD (0.05)		0.68

Table 6b. Interaction effect of MS media, sucrose (%) and IBA (mgL^{-1}) on number of roots and length of longest root.

Treatments	Number of roots	Length of longest root (cm)
Half MS+ sucrose 1.5 + IBA 1.0	3.00	5.66
Half MS+ sucrose 1.5 + IBA 2.0	3.16	4.63
Half MS+ sucrose 1.5 + IBA 3.0	3.66	9.99
Half MS+ sucrose 1.5 + IBA 4.0	3.66	4.24
Half MS+ sucrose 3.0 + IBA 1.0	4.99	8.33
Half MS+ sucrose 3.0 + IBA 2.0	4.49	6.64
Half MS+ sucrose 3.0 + IBA 3.0	3.49	4.74
Half MS+ sucrose 4.0 + IBA 4.0	3.49	4.78
Full MS+ sucrose 1.5 + IBA 1.0	1.83	2.66
Full MS+ sucrose 1.5 + IBA 2.0	2.83	4.19
Full MS+ sucrose 1.5 + IBA 3.0	2.49	5.93
Full MS+ sucrose 1.5 + IBA 4.0	4.50	2.33
Full MS+ sucrose 3.0 + IBA 1.0	8.33	8.99
Full MS+ sucrose 3.0 + IBA 2.0	5.83	4.33
Full MS+ sucrose 3.0 + IBA 3.0	3.50	2.49
Full MS+ sucrose 4.0 + IBA 4.0	4.49	1.58
CD (0.05)	1.07	1.47

Table 7. Biometric parameters of planted out plantlets (one month of planting)

Biometric parameters	One month after planting
Plantlet survival* (%)	90.00
Number of leaves*	3.40
Length of leaves* (cm)	7.00
Breadth of leaves* (cm)	2.18
Height of plant* (cm)	6.87

Total number of plants planted out - 300

*Mean of 30 plants

4.4. *In vitro* rooting

In vitro rooting of shoots developed by culturing male buds were noticed in all the treatment combinations (Table 6a).

4.4.1. Number of days taken for root initiation

The data on the number of days taken for root initiation were statistically analysed and presented in Table 6. MS media and sucrose levels had no significant effect on number of days taken for root initiation. IBA at different concentrations had significant influence on number of days taken for root initiation. IBA at 1.0 mgL^{-1} recorded least number of days taken for root initiation (5.08) which was found to be significantly superior to the other three levels.

Interaction among MS media, sucrose and IBA levels were found to be significant (Table 6a). Minimum number of days taken for root initiation (4.66) was noticed in the treatment combination of 3 per cent sucrose with IBA 1.0 mgL^{-1} in full MS medium which was on par with the same media supplements in half MS (5.16) and also combination of sucrose at 1.5 per cent with IBA 1.0 mgL^{-1} under full MS (5.16).

4.4.2. Number of roots

The data presented in Table 6 showed the influence of various treatments on number of roots.

MS media significantly affected the number of roots. Higher number of roots (4.23) were observed in full MS media and was significantly superior. Significant difference was also noticed with regard to number of roots at different levels of sucrose and IBA. Sucrose at 3 per cent was found to be significantly superior and recorded higher number of roots (4.83). When considering IBA alone, IBA at 1.0 mgL^{-1} recorded more number of roots (4.54) which was on par with IBA 2.0 mgL^{-1} (4.08) and 4.0 mgL^{-1} (4.04).

Among the different treatment combination of MS media, sucrose and IBA, sucrose 3 per cent with IBA 1.0 mgL^{-1} under full MS media recorded higher number

Plate 8. ***In vitro*** **rooting in full MS + sucrose 3.0 % + IBA 1.0 mgL**$^{-1}$

Plate 9. Acclimatized banana plantlets after one month of planting

of roots (8.33) (Plate 8) and was superior to all the other treatment combinations (Table 6b).

4.4.3. Length of longest root

The effect of MS media, sucrose and IBA levels on length of longest root is furnished in Table 4.

Addition of sucrose had no influence on length of longest root. Longest root (6.13 cm) was observed in half MS media which was significantly superior to full MS media (4.06 cm). Different concentration of IBA also had significant influence on the length of longest root. IBA at 1.0 mgL^{-1} recorded the longest root (6.41 cm) which was on par with 3.0 mgL^{-1} IBA (5.79 cm).

With regard to the length of the longest root, significant interaction was observed in combination of sucrose, MS media and IBA (Table 6b). Longest root (9.99 cm) was noted in combination with sucrose 1.5 per cent and IBA 3.0 mgL^{-1} in half MS media which was comparable with 3 per cent sucrose and 1.0 mgL^{-1} IBA in full MS media (8.99 cm) (Table 6b).

4.5. Hardening

Data on plantlet survival (%), number of leaves, length of leaves (cm), breadth of leaves (cm) and height of plant (cm) were recorded one month after planting (Table 7). After one month of planting, ninety per cent of the plantlets were survived (Plate 9).

Discussion

5. DISCUSSION

Conventionally bananas are propagated using suckers and commercially using tissue culture plants were derived from shoot tip. In *in vitro* propagation of banana, different types of explants were used of which the most commonly used explant is shoot tip and it is being practiced in many of the commercial cultivars (Kulkarni *et al.*, 2006). Male bud can also be used as a potential explant which shows no detectable somaclonal variation with very low risk of latent virus contamination (Harirah and Khalid, 2006). As compared to soil grown suckers, male inflorescences are practically less contaminated which inturn reduces the contamination rate during micropropagation techniques and also provides an opportunity to raise good quality plants after assessing the bunch characters.

Successful *in vitro* multiplication of male inflorescence from banana cultivars like Sannachenkadali, Red Banana, Njalipoovan, Virupakshi and Sirumalai belonging to South Indian tracts has been developed (Resmi and Nair, 2007; Mahadev *et al.*, 2011; Smitha *et al.*, 2014). In Kerala, one of the most demanding and highly priced banana cultivar is Kadali and it is used for offerings in temple and also for medicinal purposes. Since, the availability of planting material of Kadali is low, a refinement of the technology in which shoot tips are used for producing tissue culture plants is required.

In view of the above facts, the present investigations were carried out in the Plant Tissue Culture Laboratory at Banana Research Station, Kannara in order to standardize a protocol for mass multiplication of banana *Musa* (AA) 'Kadali' through *in vitro* male bud culture. The route adopted for *in vitro* propagule multiplication was through direct organogenesis. The results of the study are discussed in this chapter.

5.1. Standardization of establishment media

The addition of BA in the media was found to have significant influence on the percentage of cultures established. When the number of days taken for culture

establishment and percentage of cultures established were viewed together, the combination of full MS with NAA 1.0 mgL^{-1} and BA 4.0 mgL^{-1} was rated as the best, because BA 4.0 mgL^{-1} recorded highest percentage of cultures established (Fig 5.1 and 5.2). In half MS media, the quantity of salts present were found to be not enough to nourish the explants which in turn delayed the culture establishment. The choice of correct growth regulators and its use in optimum concentration gives the best result in *in vitro* propagation of *Musa* species (Krikorian, 1982). The combination of NAA and BA in the media was found to be the best for culture establishment in most of the literature (Jarret *et al*., 1985; Bhaskar, 1991; Sapheera, 2005).

The cultures were found to be completely free from any type of fungal and bacterial contamination. Each male bud was protected under bract and hence the contamination rate was very much reduced when compared to soil grown suckers (Resmi and Nair, 2007).

5.2. Standardization of multiple shoot induction media

The addition of BA alone was found to significantly influence the number of cultures developing shoots. When the number of shoots per culture, length of micro shoot and number of leaves were considered, the combination of NAA 1.0 mgL^{-1} with BA 4.0 mgL^{-1} and same quantity of NAA with BA 6.0 mgL^{-1} under full MS was considered as the best media for multiplication (Fig 5.3, Fig 5.4 and Fig 5.5). Rooting of shoots were observed at higher concentration of NAA.

Earlier studies indicated that BA along with NAA were found to give better results in shoot multiplication media (Bhaskar, 1993; Rahman *et al*., 2004; Sapheera, 2005). The number of shoots was also directly correlated to the number of leaves. The presence of active meristems on the inflorescence apices could also be used for directly inducing multiple shoots (Krikorian *et al*., 1993). In the present study, the addition of BA favoured the induction of multiple shoots from male bud explant. The rate of shoot multiplication mainly depend upon the concentration of cytokinins (Strosse *et al*., 2004). The better performance of BAP over other

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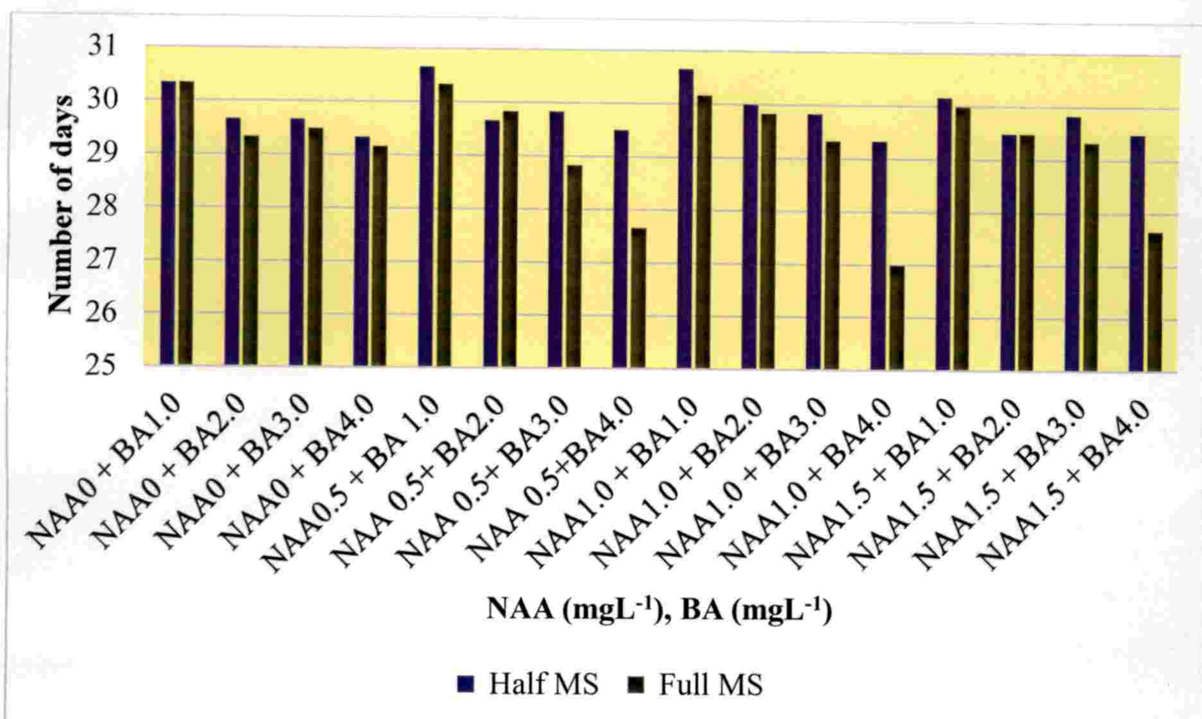

Fig 5.1. Interaction effect of combination of MS media, NAA and BA on number of days taken for culture establishment

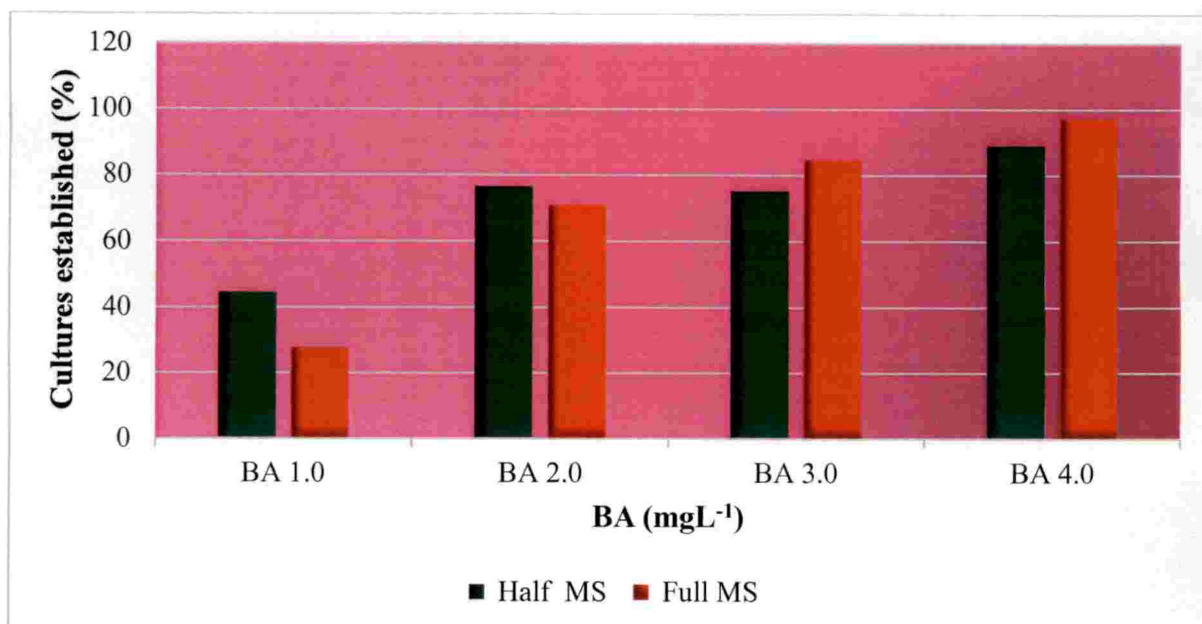

Fig 5.2. Interaction effect of MS media and BA on percentage of cultures established

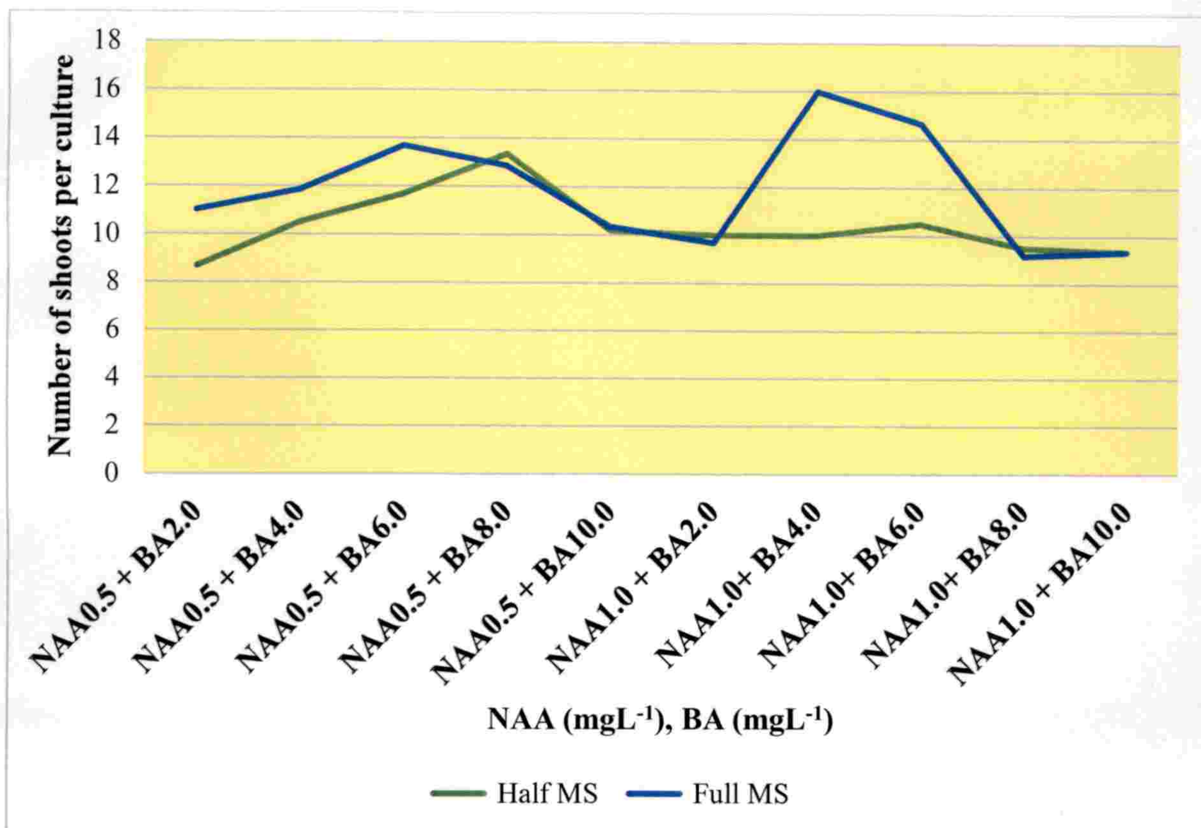

Fig 5.3. Interaction effect of MS media, NAA and BA on number of shoots per culture

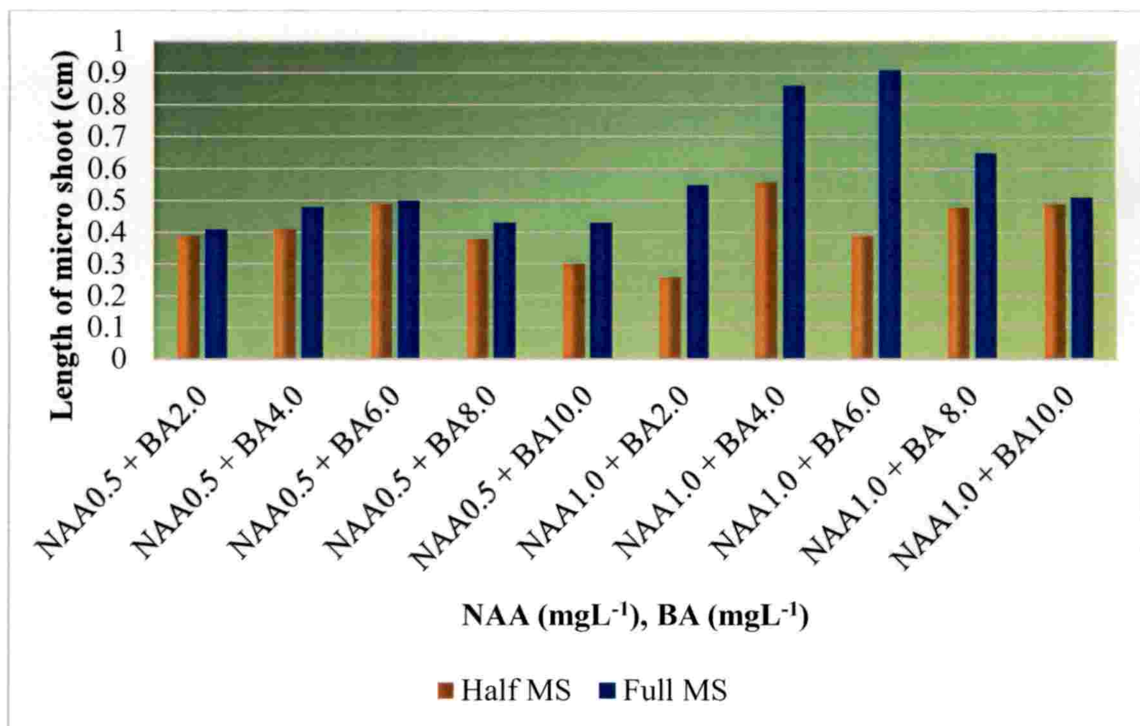

Fig 5.4. Interaction effect of MS media, NAA and BA on length of micro shoot after one month

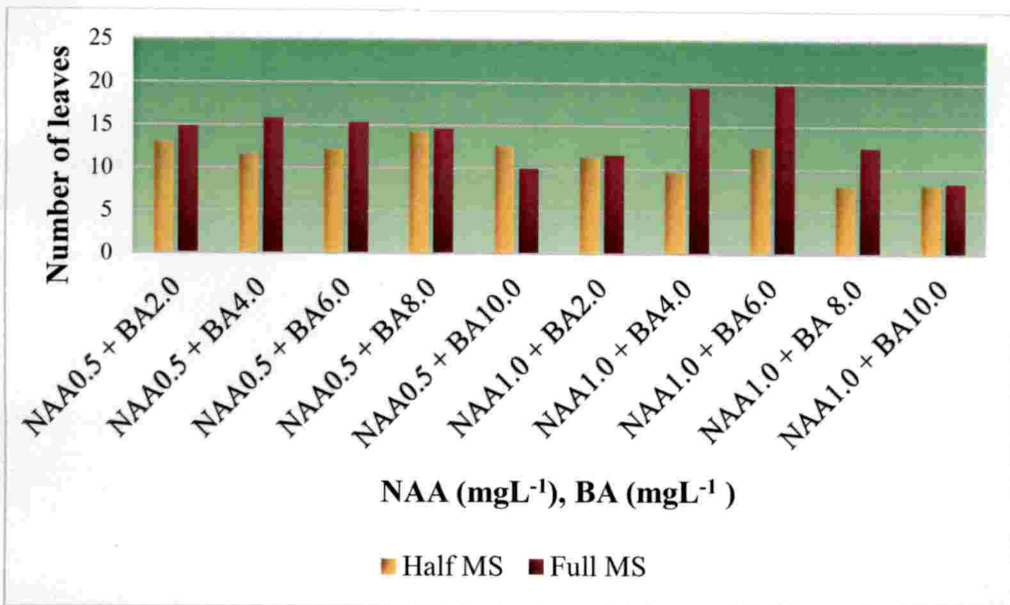

Fig 5.5. Interaction effect of MS media, NAA and BA on number of leaves

cytokinins in the multiplication of shoot tips has been reported in different cultivars of banana (Wong, 1986; Zaffari *et al*., 2000). The use of cytokinins reduce the apical dominance and induce axillary shoots (Madhulatha *et al*., 2004).

The favourable effect in inducing more number of shoots with optimum concentration of BA in male bud culture has been reported by Mahadev *et al*. (2011). Further increase in the BA concentrations drastically reduced the number of shoots in male bud culture. Increase in concentration of cytokinin beyond a limit is found to lead to a static stage of culture or results in a decrease in the proliferation of cultures (Vuylsteke, 1989). A study conducted using male inflorescence of Virupakshi and Sirumalai recorded that the best multiplication of explant was noticed in the media containing BAP 5.0 mgL^{-1}. The maximum length of micro shoot was also observed in the medium containing optimum concentration of BA. Increasing the BAP concentration beyond the optimum level resulted in a decrease in the length of the shoot (Mahadev *et al*., 2011). Buah *et al*. (2010) stated that the degree of efficiency of multiple shoot development depend upon the type of hormone and plantain cultivar and reported the development of maximum number of shoots in *Musa* spp Oniaba and Apantu pa at 4.5 mgL^{-1} BAP.

During the course of the present study, it was very evident that cytokinins played a major role in the establishment and proliferation of cultures. Auxins (NAA and IAA) at certain concentrations were also found to have influence on axillary shoot formation (Bhaskar, 1991). The presence of roots in the multiple shoot induction media is due to the addition of NAA, an auxin, which led to the induction of roots. Thus, the application of NAA can be avoided in multiple shoot induction media, as the development of roots is undesirable in a multiple shoot induction media.

5.3. Effect of media supplements

The addition of coconut water and TDZ were found to have a significant effect on number of shoots and number of leaves per culture. The best results were recorded in the full MS media containing 15 per cent coconut water and 0.4 mgL^{-1}

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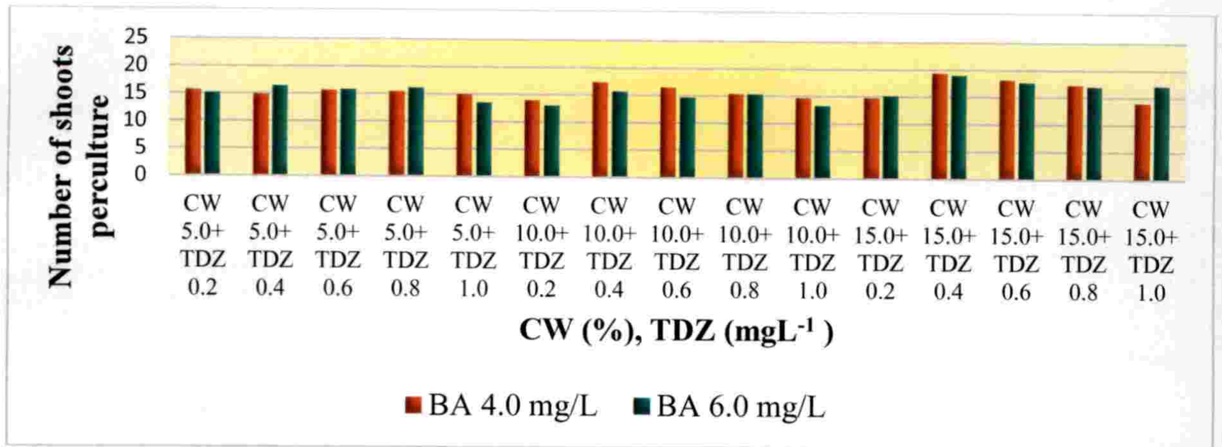

Fig 5.6. Interaction effect of media supplements on number of shoots per culture

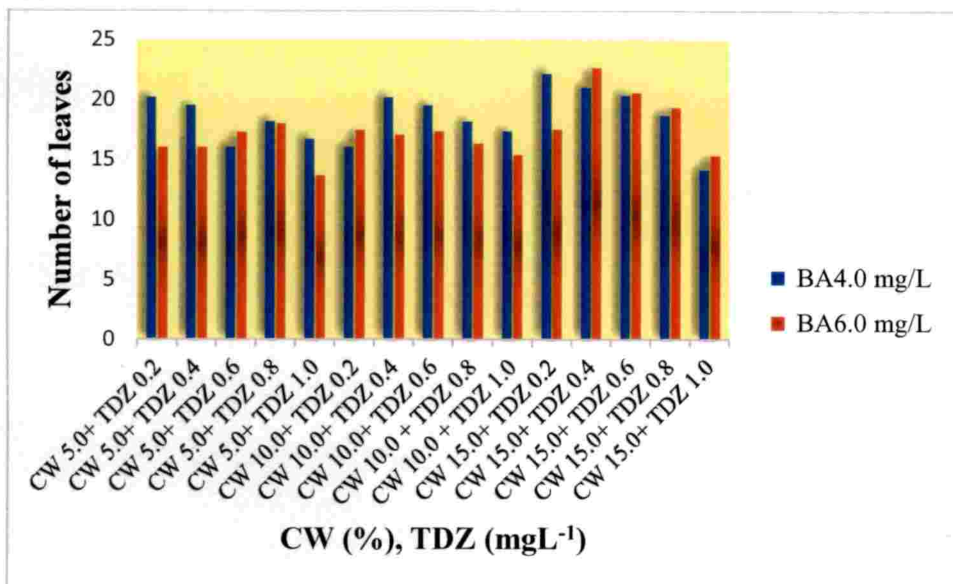

Fig 5.7. Interaction effect of media supplements on number of leaves

TDZ either in BA 4.0 mgL^{-1} or BA 6.0 mgL^{-1} (Fig 5.6 and 5.7 respectively) and was found to be better than the normal multiple shoot induction media. Length of micro shoot and induction of roots were uniform under all the combinations.

The favourable effect of BA at 4.0 mgL^{-1} supplemented with 15 per cent coconut water has been reported in multiple shoot induction using floral apex of Red banana (Bhaskar, 1991). According to Krikorian and Cronauer (1984), addition of coconut water (15 %) would be beneficial for inducing growth in banana shoot tips. The favourable effects of TDZ in multiple shoot induction of banana culture has been reported by many workers. Arinaitwe *et al.* (1999) reported that banana cultivars responded well to different concentrations of TDZ than other cytokinins. The highest multiplication rate of male bud cultures were noticed in media supplemented with TDZ as noticed in different banana cultivars (Shirani *et al.*, 2009 ; Darvari *et al.*, 2010 ; Smitha *et al.*, 2014). This experiment confirmed that modified media was better than the multiple shoot induction media as the rate of multiplication was higher in the modified media containing coconut water and TDZ.

5.4. *In vitro* rooting

De novo regeneration of adventitious roots was observed in *in vitro* rooting. A scrutiny of the results revealed that banana cv. Kadali recorded minimum number of days for root initiation (Fig 5.8) and produced higher number of roots (Fig 5.9) in full MS medium with 3 per cent sucrose and IBA 1.0 mgL^{-1}. The longest root was observed in the combination of half MS with 1.5 per cent sucrose and IBA 3.0 mgL^{-1} which was on par with 3.0 per cent sucrose and IBA 1.0 mgL^{-1} under full MS (Fig 5.10). When all the above observations were pooled together, the combination of full MS with sucrose 3 per cent and IBA 1.0 mgL^{-1} was found to be the best medium for *in vitro* rooting.

Rooting mostly occurred in full strength of MS medium but rooting in medium with half concentration of macronutrients was also suggested by Vuylsteke and De Langhe, (1985) and Novak *et al.* (1990). Sucrose has already been

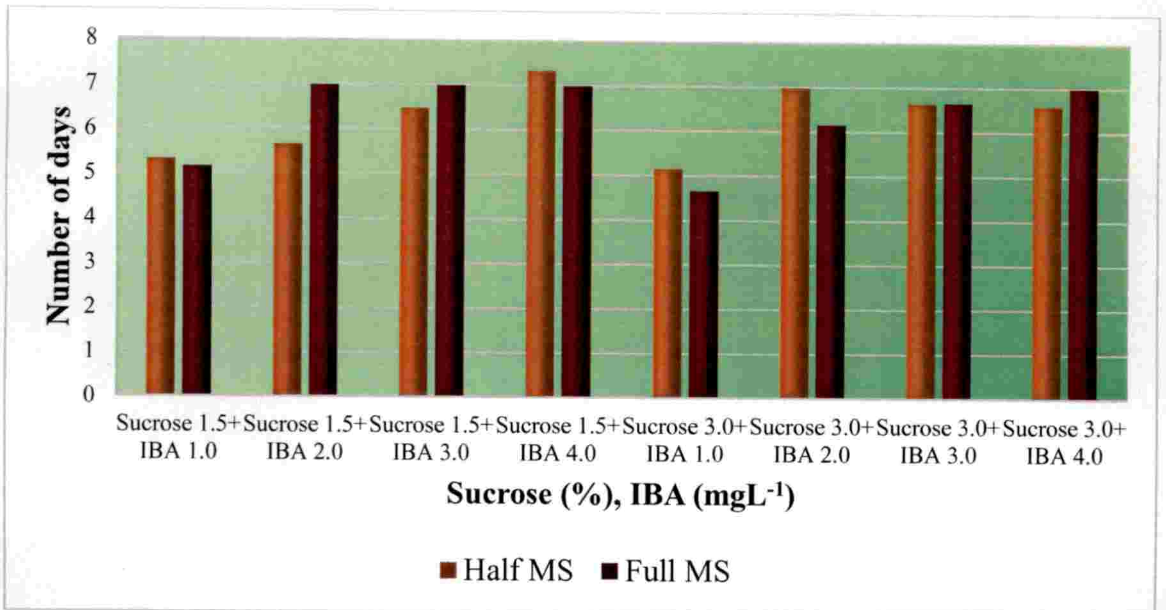

Fig 5.8. Interaction effect of MS media, sucrose and IBA on number of days taken for root initiation

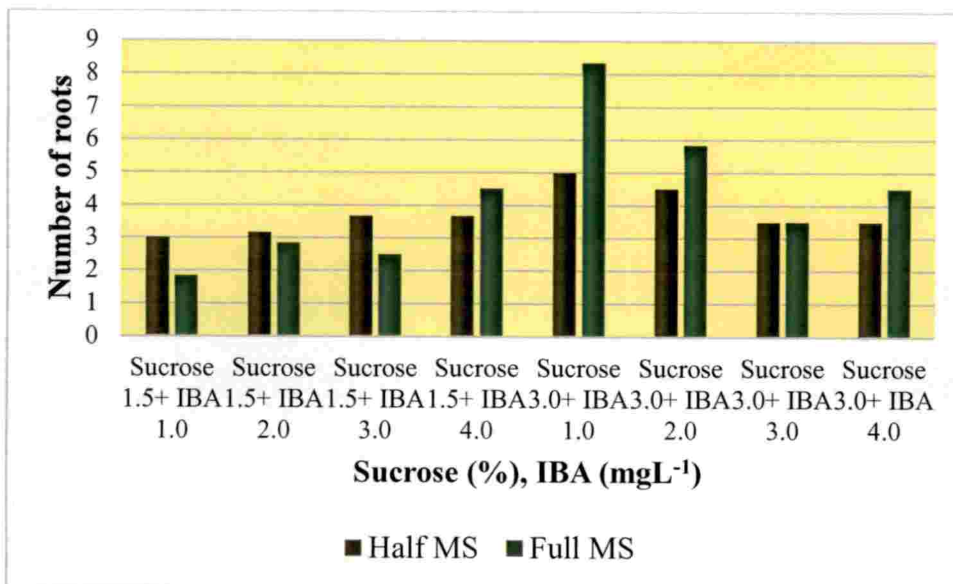

Fig 5.9. Interaction effect of MS media, sucrose and IBA on number of roots

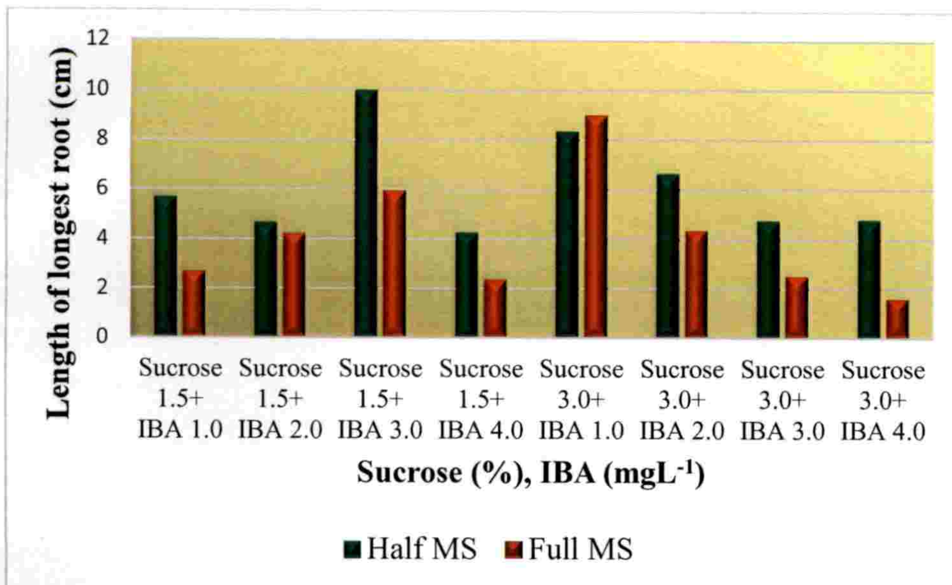

Fig 5.10. Interaction effect of MS media, sucrose and IBA on length of longest root

recognised as a source of energy as well as a factor for osmoregulation for optimising the rooting response (Chong and Pua, 1985). The favourable effects of reduction of sucrose into half of its concentration in rooting medium has been reported in banana shoot tips (Mateille and Foncelle, 1988; Bhaskar, 1991; Sapheera, 2005). A study conducted by Hussein (2012) on the effect of sucrose in MS medium in banana cultures reported an increase in plantlet fresh weight at sucrose 3.0 mgl^{-1}.

IBA at 1 μM or even at 10-50 μM were also found to be effective in rooting of *Musa* cultivars (Dore Swamy *et al.*, 1983; Mante and Tepper, 1983; Banerjee and De Langhe, 1985; Vuylsteke and De Langhe, 1985). MS medium supplemented with different concentrations of IBA is best for good rooting of banana (Raut and Lokhande, 1989; Khanam *et al.*, 1996). The results were in confirmity with a study, where different concentrations of IBA were used in rooting of male bud culture of banana cv. Sabri. The best rooting was noticed in medium supplemented with IBA 1.0 mgl^{-1} (Sultan *et al.*, 2011).

5.5. Hardening

Acclimatization is an essential and very necessary process for preparing the *in vitro* grown plantlets before planting out in the field as they are very sensitive and require more time to get adapted to the external conditions (Brainerd and Funchigmi, 1981). Well rooted plants were hardened after potting in pro trays containing 1:1 (v/v) sterilized coco peat and vermiculite. After one month of planting out, 90 per cent of the plants were found to survive. In this technique, one male bud provided an average of 10 explants and each explant under *in vitro* conditions produced an average of 16 shoots within a period of three months time. Thereby within a span of 90 days, on an average, a total number of 160 shoots were found to develop from a single male bud, which can either be induced to produce more number of shoots or can be allowed to develop into plantlets. Hence, male bud culture can be efficiently used in addition to shoot tip culture to produce large number of planting material of this elite genotype to meet the present day demand of farming community.

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Tissue culture plantlets just transferred to external conditions are photosynthetically inefficient and so weekly sprays of inorganic salt solution is required for the growth of plant (Bhaskar, 1991; Sundararasu, 2003; Sapheera, 2005). The standard mist chamber was proved to be the best hardening unit for maximum survival of banana plantlets (Sapheera, 2005). Successful hardening of tissue culture plantlets depends upon the potting medium used, hardening units, containers used and foliar application of nutrient solutions (Bhaskar, 1991; Sundararasu, 2003; Sapheera, 2005).

The present study demonstrated that *in vitro* male bud culture of Kadali can provide disease free plantlets. Therefore this protocol can be applied for the mass multiplication of Kadali (AA) whereby the huge demand for good quality, disease free planting material can be fullfilled. Moreover, it could be of great help in many cases where suckers of elite cultivars are scarce.

In vitro male bud culture is a cost effective method and also has an advantage of the absence of latent contamination (Harirah and Khalid, 2006), which is often faced during the tissue culture protocols which are currently being used for commercial cultivation of banana. One male bud can provide an average of 10 explants and each explant can provide an average of 16 shoots. Hence, male bud culture can be used to meet the great demand of large number of suckers of elite genotypes.

As compared to suckers of Kadali, the number of multiple shoots produced in *in vitro* is less in male bud culture. Hence the possibilities for enhancing the rate of multiplication by adding different media supplements has be explored. Field studies on the morphological, flowering and fruiting characters of the planted out plantlets has to be conducted.

Summary

6. SUMMARY

Investigation on "Standardization of *in vitro* male bud culture in banana *Musa* (AA) 'Kadali" was carried out in the Plant Tissue Culture Laboratory at Banana Research Station, Kannara during the period 2016-2018. The results of the study are summarised below.

1. For the better and speeder establishment of male bud explants, full MS medium containing NAA 1.0 mgL^{-1} and BA 4.0 mgL^{-1} was found to be the best.
2. Culture establishment was found to be influenced by the combination of MS media and BA concentration.
3. Multiple shoot induction in cultures were more rapid in full MS media rather than in half MS media.
4. Presence of BA in the media was found to significantly influence the development of shoots in culture. Cent per cent cultures developed shoots in BA 4.0 mgL^{-1} and BA 6.0 mgL^{-1}. Combination of MS media with NAA and BA had no significant influence on cultures developing shoots.
5. Among the different combinations, the number of shoots produced was significantly higher (15.99) in the medium containing NAA 1.0 mgL^{-1} and BA 4.0 mgL^{-1} in full MS.
6. In multiple shoot induction media, maximum length of micro shoot (0.91 cm) after one month was observed in full MS medium supplemented with NAA 1.0 mgL^{-1} and BA 6.0 mgL^{-1} and was found to be comparable with the same medium containing BA 4.0 mgL^{-1} (0.86 cm).
7. A differential response was noticed in MS medium supplemented with NAA and BA with respect to number of leaves. Full MS medium containing NAA 1.0 mgL^{-1} and BA 6.0 mgL^{-1} was found to be the best for production of maximum number of leaves (19.83) which was on par with the cultures supplied with NAA 1.0 mgL^{-1} and BA 4.0 mgL^{-1} in full MS medium (19.50).

8. Maximum number of roots (18.07) were observed in half MS medium supplemented with NAA 1.0 mgL^{-1} and BA 2.0 mgL^{-1} and was comparable with half MS containing NAA 0.5 mgl^{-1} and BA 2.0 mgL^{-1} (15.11). Higher concentrations of BA suppressed root growth in multiplication media.
9. The best media found to be suitable for multiplication of shoots was full MS supplemented with NAA 1.0 mgL^{-1} and BA 4.0 mgL^{-1} and also the same media combination but with higher BA level (BA 6.0 mgL^{-1}).
10. When the media identified as the best media for multiple shoot induction was modified by adding coconut water and TDZ, it resulted in cent per cent proliferation of cultures.
11. Full MS media with BA 4.0 mgL^{-1}, 15 per cent coconut water and 0.4 mgL^{-1} TDZ recorded maximum number of shoots (19.38) and was found to be on par with the combination of BA 6.0 mgL^{-1} with same amount of coconut water and TDZ (18.99).
12. When the media found to be the best for multiple shoot induction was compared with the modified media, maximum number of shoots were recorded in modified media. But economically modified media was found to be costlier.
13. Of the various combinations tried, BA 4.0 mgL^{-1}, 15 per cent coconut water and 0.4 mgL^{-1} TDZ recorded maximum number of leaves (22.66), which was on par with the combination, BA 4.0 mgL^{-1}, coconut water 15 per cent and 0.2 mgL^{-1} TDZ (22.16). Length of micro shoot and number of roots did not show any significant difference with respect to different media combination.
14. Minimum number of days for root initiation (4.66) were recorded in full MS medium with 3 per cent sucrose and 1.0 mgL^{-1} IBA and was found to be on par with the same media supplements in half MS (5.16) and also for full MS in combination with sucrose at 1.5 per cent and IBA 1.0 mgL^{-1} (5.16).

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15. Significantly higher number of roots (8.33) were recorded in full MS medium with 3 per cent sucrose and 1.0 mgL^{-1} IBA.
16. Longest root (9.99 cm) was noted in half MS medium in combination with sucrose 1.5 per cent and IBA 3.0 mgL^{-1} and was comparable with full MS media containing 3 per cent sucrose and 1.0 mgL^{-1} IBA (8.99).
17. After the hardening stage, ninety per cent of the plantlets planted out were found to survive after one month of planting.

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* Originals not seen

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Appendix

Appendix

Composition of Murashige and Skoog Medium (1962)

Major elements	mgL^{-1}
$CaCl_2.2H_2O$	440.0
$FeSO_4.H_2O$	27.8
KNO_3	1900.0
KH_2PO_4	170.0
$MgSO_4.7H_2O$	370.0
NH_4NO_3	1650.0
$Na_2.EDTA$	37.5
Minor elements	
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
H_3BO_3	6.20
KI	0.83
$MnSO_4$	22.30
$NaMoO_4.2H_2O$	0.25
$ZnSO_4$	8.6
Organic constituents	
Glycine	2.0
Myo-inositol	100.0
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Sucrose	30.0 g
Agar	7.0 g

STANDARDIZATION OF *IN VITRO* MALE BUD CULTURE IN BANANA *MUSA* (AA) 'KADALI'

By

LAKSHMI K. S.

(2016-12-013)

ABSTRACT OF THE THESIS

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ABSTRACT

The experiment entitled "Standardization of *in vitro* male bud culture in banana *Musa* (AA) 'Kadali'" was taken up with the objective of standardizing a protocol for mass multiplication of banana *Musa* (AA) 'Kadali' through *in vitro* male bud culture. The work was carried out in the Plant Tissue Culture Laboratory at Banana Research Station, Kannara during 2016-2018. Active meristems of male flowers were used as the explant. The study was undertaken in three parts (1) standardization of establishment media (2) standardization of multiple shoot induction media (3) *in vitro* rooting and acclimatization.

The results indicated that full MS medium containing a combination of NAA 1.0 mgL^{-1} and BA 4.0 mgL^{-1} was suitable for better and faster explant establishment. In multiple shoot induction media, multiple shoots were found to develop in all the cultures with full MS medium containing BA 4.0 mgL^{-1} and BA 6.0 mgL^{-1}, and the highest number of shoots were recorded in full MS medium supplemented with NAA 1.0 mgL^{-1} + BA 4.0 mgL^{-1}. Considering the length of micro shoots and number of leaves, full MS + NAA 1.0 mgL^{-1} + BA 6.0 mgL^{-1} and full MS + NAA 1.0 mgL^{-1} + BA 4.0 mgL^{-1} were found to be the best.

The best media identified from multiple shoot induction cultures (full MS+ NAA 1.0 mgL^{-1} + BA 4.0 mgL^{-1} and full MS + NAA 1.0 mgL^{-1} + BA 6.0 mgL^{-1}) were modified by adding different levels of thidiazuron (TDZ) and coconut water (CW). Highest number of shoots were observed in full MS + NAA 1.0 mgL^{-1} + BA 4.0 mgL^{-1} + CW 15 % + TDZ 0.4 $mgL^{-1}$and full MS + NAA 1.0 mgL^{-1} + BA 6.0 mgL^{-1}+ CW 15 % + TDZ 0.4 mgL^{-1}. With regard to number of leaves, full MS + NAA 1.0 mgL^{-1} + BA 6.0 mgL^{-1} + CW 15 % + TDZ 0.4 $mgL^{-1}$and full MS + NAA 1.0 mgL^{-1} + BA 4.0 mgL^{-1}+ CW 15 % + TDZ 0.2 mgL^{-1} recorded the highest value. Length of micro shoots did not show any significant difference.

In *in vitro* rooting studies, early rooting and significantly higher number of roots were recorded in full MS medium with three per cent sucrose and IBA 1.0 mgL^{-1}. Root length was highest in the combination of half MS with 1.5 per cent sucrose and IBA 3.0 mgL^{-1}.

Well rooted plants were hardened after potting in pro trays containing 1:1 (v/v) sterilized coco peat and vermiculite. After one month of planting out, 90 per cent of the plants were found to survive.

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