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Optimizing the effect of plant growth regulators on *in vitro* micro propagation of Indian red banana (*Musa acuminata*)

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Abstract
Red banana is an important fruit crop of India. The micro propagation of shoot tips *in vitro* is a widespread application of biotechnology in agriculture. Micro propagation is advantageous over conventional techniques traditional propagation, opens fresh avenues towards conservation of endangered species, disease free plants, rapid and large scale production of elite plants all year round. Therefore, the current investigation emphasizes the effect of plant growth regulators on the micro propagation as well as on the biochemical estimation of red banana. The meristem tip was used as explants. Among the tested combinations and concentrations 3 mg/l BAP + 0.2 mg/l NAA showed highest shooting and 2 mg/l IBA with 0.5 and 1 mg/l IAA also. The highest root length and no. of roots was observed with the 1 mg/l IBA. Subsequently, the micro propagated plants were hardened in green house followed by the acclimatization. Significant increase in chlorophyll, carbohydrate, protein and phenol content was observed. These findings concluded that a fruitful and practicable protocol was set up for the successful regeneration of new plantlets of red banana. Micro propagation of red banana is beneficial further in somaclonal variation, genetic transformation and secondary metabolite production. The nutritional and medicinal potential of red banana is well documented which can be practiced as an alternative remedy for treating certain ailments.

Keywords: Red banana, Micro propagation, Plant growth regulators, Sword suckers

Introduction
The word banana is a general term embracing a number of species or hybrids in the genus Musa of the family Musaceae. Bananas are the most important and most widely grown fruit crops in India (Al-Amin *et al.*, 2009; Kahia *et al.*, 2015; Lohidas and Sujin, 2015)) [1-3]. Banana, fourth most important food commodity on earth and the second largest food-fruit crops of the world has great socio-economic importance in India (Aquil *et al.*, 2012) [4]. Bananas are a great source of potassium, and are accompanied by their signature sweet taste, but they have a cousin that tastes even sweeter. Many of us are less familiar with the red bananas or Red Dacca banana with reddish-purple skin (Fig 1). The red bananas are smaller, plumper, softer and have a unique flavour, similar to yellow, but mixed with the taste of raspberries. When ripe, raw red bananas have flesh that is cream to light pink in colour (Rajoriya, 2011) [5].

The redder the fruit more nutritious elements it contains. Though red bananas are rare but when it comes to vitamin C and potassium, they are more affluent than yellow ones. On an average one banana contains 15% of the vitamin C, 11% of the potassium and 16% of the dietary fibre needed each day for good health (Rajoriya, 2011) [5]. They also offer a number of health benefits such as strengthens immune system, relieves heart health, improves eye and skin health, alleviates digestive problems and helps combat smoking (Rajoriya, 2011) [5]. They also contain a rich supply of beta carotene, vitamin B_6. They have no fat, cholesterol or sodium and contain more digestible carbohydrates than any other fruit (Al-Amin *et al.*, 2009) [1]. Red bananas can be eaten plain, sliced into fruit salads, included in baked goods, dried to make banana chips or fried as a side dish. Banana leaves can be used as eco friendly, healthy and hygienic eating plates. Banana fibres can be used to make handicrafts items like bags, pots and wall hangers. Good quality paper and rope can be prepared from banana waste (FAO STAT, 2014) [6].

Fig 1: Red Bananas in natural condition

Bananas are best cultivated in a highly organic soil, with a neutral to slightly acid pH (5.5–7.0). A lot of water is usually needed for the growth and yield of bananas. Since this herb is sterile, the only way of propagating by the removal of suckers from the original plant (Anonymous, 2012) [7]. India is the largest producer of banana in the world however, incapable of exporting a substantial amount mainly due to post harvest problems (Anonymous, 2013) [8].

More specifically, shortage of planting material and synchronisation of fruit ripening are two major problems that cause unavoidable trouble to farmers, it is almost impossible to export fresh bananas. The production of disease free planting material of banana by meristem culture technique is an urgent need. Thus, there is a need to establish a micro propagation protocol for the banana cultivar not only for propagation, multiplication and preservation but also for enhancement of secondary metabolites (Anonymous, 2002) [9]. As several researchers have reported the regeneration of Musa spp. via micro propagation (Nguyen and Kozai, 2001; Madhulatha *et al*., 2004; Uzaribara *et al.*, 2015; Noor *et al.*, 2017 Singh; *et al.*, 2017) [10-14].

Plant tissue culture is a form of biotechnology with great potential to aid ability to regenerate a whole plant (totipotency), has become an important tool-set for modern day biotechnology. Micro propagation has played a key role in banana and plantain breeding programs worldwide (Rowe and Rosales, 1996; Vuylsteke *et al*., 1997) [15, 16]. Since, micro propagation through tissue culture is known to have potential to produce rapidly a large number of plants and also free from pathogens, in a short time and space. Micro propagation has been carried out in several crops at ICAR which include potato, banana, ginger, mango and several other medicinal and aromatic plants, coffee, tea, citrus varieties with disease free planting material have also been produced. Coconut, banana, oil palm, ornamental plants have been multiplied and commercialized by private seed companies (Anonymous, 2007; Anonymous, 2012; Anonymous, 2013) [17, 7, 8]. Over a million of plant species can be grown from a small or even a microscopic piece of plant tissue within a year (Mantell *et al.*, 1985; Vasil, 1991; Dadjo *et al.*, 2014) [18-20]. Banana plantlets regenerated through tissue culture have higher survival rate, reduce the cost of disease and pest control, show vigorous growth and have a shorter harvesting period with a great practical and economic benefits (Israeli *et al.*, 1995; Ortiz and Vuylsteke, 1994) [21, 22]. In this contest, the current study was undertaken to minimize the above mentioned problems with the major objective to develop a protocol for *in vitro* micro propagation followed by the effect of plant growth regulators (PGRs) and thereafter biochemical estimation of regenerated plantlets of red banana.

Materials and Methods

Collection of explant and chemicals

Healthy three months old sword suckers of the red banana used as an explant were collected from the field grown plants at Sam Higginbottom University of Agriculture, Technology & Sciences, Allahabad. Chemicals used in the preparation of MS media and biochemical estimation were procured from Merck and Hi media.

Sterilization of explant

After removing the outer sheaths of the suckers, they were cut into 5 to 10 cm of pieces. These suckers were kept under running tap water (for 30 mins.) followed by the few drops of Tween-20 (surfactant for 5 to 10 mins.) and fungicides like bavestin or indophil (0.1% for 2 to 5 mins.). They were washed thoroughly with sterile distilled water (4 to 5 times) so that no remaining chemical residue. After sterilization the explants were kept under the laminar flow chamber and the UV lights switched on to ensure the pathogen free ambience for have been were sterilized with 80% sodium hypochlorite followed by 0.1% mercuric chloride and 80% ethanol. After these treatments these explants were rinsed with sterile distilled water and kept to dry for few minutes and trimmed up to 3 to 5 cm.

Choice of culture media and culture conditions

Establishment of culture for the initial shoot initiation MS basal media (Murashige and Skoog, 1962) [23] was supplemented with different combinations of BAP (6-Benzyl amino purine) and NAA (1-Naphthalene acetic acid). For the initiation and growth of roots in the asceptic culture MS basal media was supplemented with different combinations of IBA (3-Indole butyric acid) and IAA (Indole-3-acetic acid). The pH was maintained to 5.8 prior to addition of gelling agent agar then the media was autoclaved at 120°C and 15 *p.s.i* pressure. The sterile explants were culture onto medium and the cultures were kept at 28˚C and a photoperiod of 16 hrs light intensity. Visual observations like number of days taken for shoot initiation, shoot length, root length and other similar parameters were recorded regularly.

Biochemical estimation of *in vitro* regenerated plantlets of red banana

For all the biochemical estimation leaves of regenerated plants were used and the whole experiments were repeated in triplicate.

Estimation of chlorophyll

The method of Rastogi and Dwivedi (2006) [24] was followed to estimate chlorophyll a, chlorophyll b and total chlorophyll contents. 1 g of leaf sample was weighed, finely cut and homogenized with 20 ml of pure acetone. The extract was filtered through Whatman No. 1 filter paper and washed 2 to 3 times using 80% acetone. Finally, the volume of the extract was made up to was made upto 25 ml. The absorbance of the extract was read at 645 and 663 while for blank, 80% acetone was used. The amount of chlorophyll present in the extract (mg chlorophyll per g tissue) was calculated using the following equations:

mg chlorophyll (a/g tissue)=12.7(A663)-2.69(A645) x V/1000 x W

mg chlorophyll (b/g tissue)=22.9(A645)-4.68(A663) x V/1000 x W

mg chlorophyll (per g tissue)=20.2(A645)+8.02(A663) x V/1000 x W

where, A= Absorbance at specific wavelengths, V= Final volume of chlorophyll extract in 80% acetone and W=Fresh weight of the tissue extracted.

Estimation of carotenoid

The method of Lichtenthaler and Welburn (1983**)** [25] was used to determined carotenoid. Fresh leaves weighing of 0.5 gm were taken and homogenized in 10 ml of acetone (80% acetone) and centrifuged at 3000 rpm for 10 min, then supernatant was collected. The absorbance of was recorded at 470 nm. It is calculated by using the formula given below: Total carotenoids = [1000 A470 – (2.270 Chl-a - 81.4 Chl-b)]/227

Estimation of Carbohydrate

Anthrone method was used for total carbohydrates were determined given by Hedge and Hofreiter (1962) [26]. Briefly, 100 mg of the plant leaf sample was hydrolysed with 5 ml of 2.5 N HCl for hours in a boiling water bath, cooled at room temperature and neutralized with sodium carbonate. The volume was made upto 100 ml and centrifuged to collect supernatant. The working standard serves as blank. To the 1 ml of supernatant, 4 ml of anthrone reagent was added and heated for eight minutes in a boiling water bath. Now rapidly cooled it and read the green to dark green colour at 630 nm using spectrophotometer. A standard graph by plotting concentration of the standard and the carbohydrates was calculated in terms of mg/g.

Estimation of protein

Total protein was determined according to the method given by Lowry *et al*. (1951) [27]. 0.5 g of the leaf sample was grinded and the supernatant was collected after centrifugation. 5 ml of alkaline copper sulphate reagent along with distilled water was added to the supernatant then incubated at room temperature for 10 min. Now, 0.5 ml of Folin Ciocalteau reagent (FCR) was added to the obtained solution and incubated at room temperature in dark for 30 min. The absorbance at 660 nm was measured in a spectrophotometer after colour development. A standard graph was plotted against the absorbance; the amount of protein was calculated in terms of mg/g.

Estimation of phenol

Determination of phenol was done accordingly with Bray and Thorpe (1954) [28] method. Leaf sample was centrifuged in 80% ethanol for 10 minutes. The supernatant collected and the residue was re-extracted and centrifuged five times using 80% ethanol. The supernatant thus collected was evaporated till dryness and the dried residue was then dissolved in 5 ml of distilled water. To the obtained solution 0.2 ml solution, 2.8 ml distilled water and 0.5 ml FCR solution was added. The solution was incubated for 3 mins and then 2 ml of 20% Na_2CO_3 was added. Now the solution was boiled for 1 min, cooled and then absorbance at 650 nm was measured using spectrophotometer.

Results

Red bananas are one of the varieties of bananas that fetches more medicinal as well as nutritional values. In the present investigation, sword suckers of red banana were selected for *in vitro* studies. This study primarily aims at developing an exclusive easy and rapid protocol for micro propagation of red banana.

Effect of PGRs on shoot regeneration

The concentration and combination of auxin and cytokinin in culture media is a key factor which determines successful shoot regeneration. After sterilization the suckers were inoculated on MS medium supplemented with concentrations of BAP and NAA to determine their effect on the shoot initiation. The results are depicted in the Table 1 and portrayed in Fig 2. Among different combinations analyzed, MS medium supplemented with 3.0 mg/l of BAP and 0.2 mg/l of NAA of medium showed good results both for shoot initiation and multiplication.

Table 1: Effect of PGRs on shoot proliferation and multiplication of *in vitro* regenerated plants of red banana

Treatment	PGRs (mg/l)		Growth Response			
	BAP	NAA	No. of explants inoculated	Shoot emergence (%)	No. of shoots per explant	Shoot length (cm)
SM 1	1	0	4	50	1	4
SM 2	1	0.2	5	50	1	4
SM 3	2	0	4	70	2	3
SM 4	2	0.2	5	75	1	5
SM 5	3	0	4	80	4	6
SM 6	3	0.2	5	90	5	9
SM 7	4	0	4	65	1	4
SM 8	4	0.2	5	60	1	5

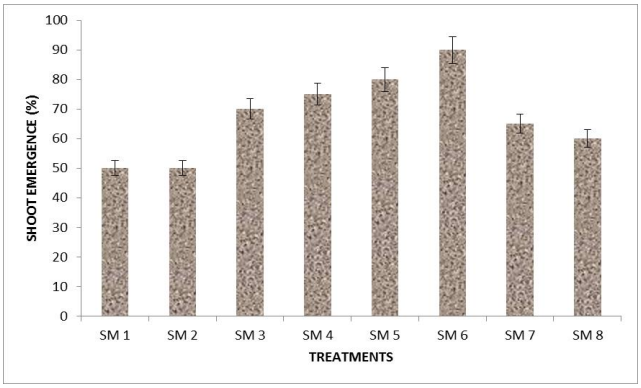

Fig 2: Effect of PGRs on shoot emergence using meristem of red banana

Effect of PGRs on rooting and hardening

The *in vitro* regenerated shoots were excised from the and onto the MS medium supplemented with different combination of IBA (1, 2 and 3 mg/l) and IAA (0.5 and 1 mg/l) for the root induction as depicted in Table 2 and Fig 3. The observations were recorded on the basis of no. of roots, rooting percentage and mean length of roots (cm). Rooting was observed within 2 to 3 weeks of culture. Among the various combinations of PGRs used, the highest rooting frequency (100%) was achieved with the 2 mg/l IBA with 0.5 and 1 mg/l IAA also. The highest root length and no. of roots was observed with the 1.0 mg/l IBA.

Root numbers varied with different concentrations of IBA and IAA. The well rooted *in vitro* raised plantlets were

successfully transplanted in the potting medium, acclimatized in the culture room and then transferred to green house after 30 days.

Effect of PGRs on the biochemical estimation of *in vitro* regenerated plants of red banana

The effect of different concentrations of PGRs on the pigment as well as metabolite content was studied after 15 days interval. Results, as depicted in Table 3 and portrayed in Fig 5 showed that the prominent rise in the pigments content *viz*. total chlorophyll and carotenoid was observed highest *i.e.* 2.19 mg/g and 0.69 mg/g respectively after 15 days. The metabolites content *viz*. carbohydrates, protein and phenol was observed highest *i.e.* 47.28 mg/g, 17.93 mg/g and 60.77 mg/g respectively after 45 days.

Table 2: Effect of PGRs on rooting of *in vitro* regenerated shoots of red banana

Treatment	PGRs (mg/l)		Growth Response		
	IBA	IAA	No. of roots	Rooting (%)	Root length (cm)
RM 1	1	0	11	53	8
RM 2	1	0.5	3	62	5
RM 3	1	1	2	75	3
RM 4	2	0	5	100	3
RM 5	2	0.5	7	100	3
RM 6	2	1	9	100	4
RM 7	3	0	4	80	3
RM 8	3	0.5	6	94	3
RM 9	3	1	7	96	3

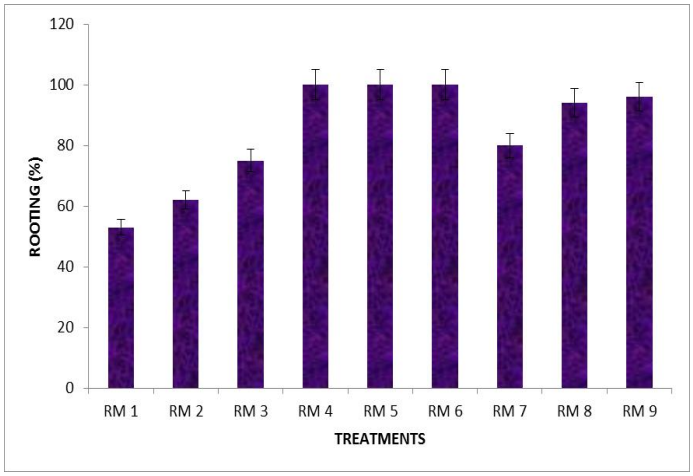

Fig 3: Effect of PGRs on rooting of *in vitro* regenerated shoots of red banana

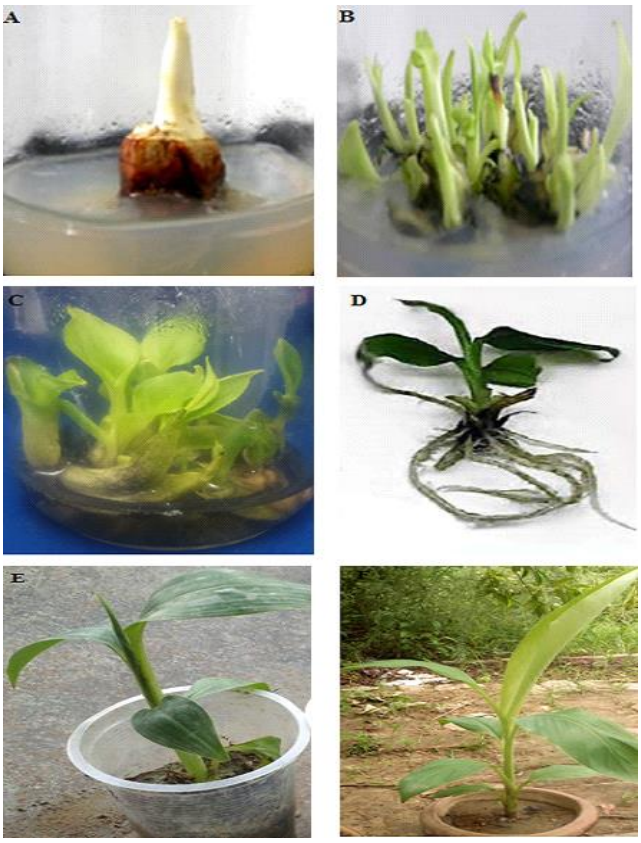

Fig 4: Micro propagation stages of meristem tip culture of red banana:
A) Initiation, B) Proliferation, C) Multiplication, D) Rooting of *in vitro* shoots, E) Hardening of *in vitro* rooted plants and F) Acclimatization in green house.

Table 3: Effect of PGRs on the biochemical estimation of *in vitro* regenerated plantlets of red banana

No. of days	Pigments (mg/g)		Metabolite content (mg/g)		
	Total Chlorophyll	Carotenoid	Carbohydrates	Protein	Phenol
15	2.19	0.69	26.64	16.22	40.64
30	1.95	0.66	35.88	17.85	49.65
45	1.92	0.65	47.28	17.93	60.77

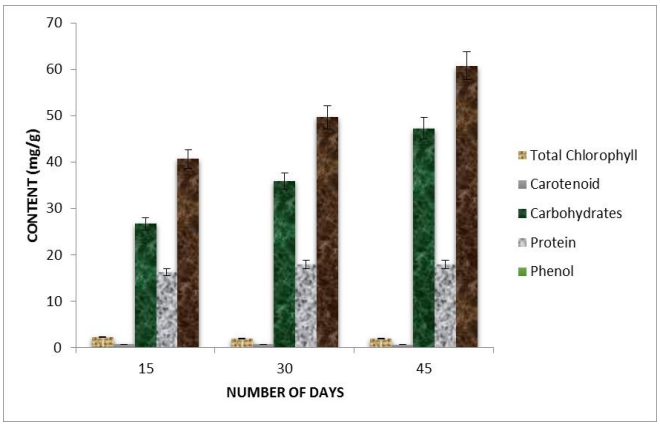

Fig 3: Effect of PGRs on the biochemical estimation of *in vitro* regenerated plantlets of red banana

Discussion

PGRs play a vital role in the regeneration of plantlets through different *in vitro* culture techniques, as they influence different plant processes comprising mostly of growth, differentiation and development (Hobbie, 1998) [29]. In this

investigation BAP and NAA were the most effective PGRs for the shoot initiation and proliferation. Our results are in good agreement with Mendes *et al.* (1999) [30] and Noor-Aziah and Khalid (2002) [31] revealed that the higher concentration of BAP gave rise to the high shoot regeneration and shoot length. Cytokinins namely BAP, Kinetin and Zeatin were needed in the culture to improve the cell differentiation, proliferation of shoot and morphogenesis (Smith, 1992; Chugh *et al.*, 2009) [32, 33].

IBA is known to plays an important role in the formation and development rooting. Root formation and plant regeneration with IBA has been reported by Agastian *et al.* (2006) [34] and Naika and Krishna (2008) [35]. Our results revealed that the high concentration of IBA was effective for root induction and root length. The root induction and elongation response to IAA were better, these findings are contrary to those of Vuylsteke (1989) [36] who reported that NAA was more effective than IAA in banana tissue culture.

The critical hormonal substance used in a nutrient media are auxins, cytokinins and gibberellins. Adenine-based cytokinins are commonly used in several banana (*Musa)* species for *in vitro* propagation and BAP is the most commonly preferred cytokinin, (Vuylsteke, 1989; Madhulatha *et al*., 2004; Venkatachalam *et al*., 2007; Bairu *et al*., 2008; Jafari *et al*., 2011) [36, 11, 37-39]. Generally, cytokinin helps in shoot proliferation and auxins helps in rooting of proliferated shoots (Al-Amin *et al.*, 2009) [1]. Many of the previous reports on banana micro propagation used more than one type of media for initiation, multiplication and rooting (Doreswamy *et al*., 1983; Cronauer and Krikorian 1984; Jarret, 1985; Cronauer and Krikorian, 1986; Diniz *et al*., 1999; Arinaitwe *et al*., 2000; Nguyen and Kozai, 2001; Gallez *et al*., 2004; Gubbuk and Pekmezei, 2004; Sheidai *et al.* 2009; Olivia *et al.*, 2010; Shirani *et al.*, 2011; Munguatosh *et al*., 2013; Reddy *et al.*, 2014; Gebeyehu, 2015; Kahia *et al.*, 2015; Kumari and Misra, 2016; Noor *et al.*, 2017) [40-45, 10, 46-53, 2, 54, 13].

The increase in the content of various biochemical parameters of *in vitro* regenerated plants may be due to the effect of different phytohormones in *in vitro* raised plants (Mohapatra *et al*., 2008) [55]. Noticeable upsurge was observed in pigment contents as well as in metabolite content of *in vitro* regenerated plants of red banana (Rajoriya *et al*., 2012a; 2012b) [56, 57]. Photosynthetic pigments mainly constitute of chlorophyll a, b, total chlorophyll and carotenoid are of vital importance in photosynthesis changes. The concentration of phenolic compounds may vary in different genotypes of the same species grown under different climatic conditions. Phenolic compounds have been shown to be involved in providing resistance of some host plants to pathogens (Nicholson and Hammerschmidt, 1992) [58].

Conclusion

On the basis of observations made and results obtained it can be concluded that the efficient and highly reliable protocol has been developed of red banana. The best medium for the shoot regeneration was supplemented with 3 mg/l BAP, 0.2 mg/l NAA and produced quality shoots and 2 mg/l IBA with 0.5 and 1 mg/l IAA rooting. On the other hand, biochemical analysis indicates productive performance. Micro propagaton of great importance as it yields a large number of plantlets within a short span. Recent advances in biotechnology for crop improvement have a great impact on banana cultivation. There has been extensive use of plant tissue culture in micro propagation of plants of forestry, horticultural and medicinal importance.

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