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Influence of Mineral Nutrients on the *in Vitro* Rooting of Micro-shoots and Hardening in Banana (*Musa paradisiaca* L.) Variety 'Grand Naine'

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ABSTRACT

To standardize a commercially viable *in vitro* rooting procedure for banana (*Musa paradisiaca* L.), micro-shoots from three week-old cultures of variety 'Grand Naine' were inoculated onto four different mineral salt concentrations of MS (Murashige and Skoog) media supplemented with 2 mg l^{-1} NAA (α-naphthyleneacetic acid), 20 g l^{-1} sucrose, 100 mg l^{-1} activated charcoal and 8 g l^{-1} agar. After three weeks of incubation, the micro-shoots cultured onto ¾-strength MS media showed the best response in terms of root initiation and growth. After hardening, plantlets developed on ½- or ¾-strength MS medium could be acclimatized and grew well *ex vitro*. Therefore, low mineral salt concentration in the *in vitro* rooting media can be adopted as an effective low-cost *in vitro* rooting procedure for banana 'Grand Naine'.

Keywords: *ex vitro* growth, commercially viable method, micropropagation, nutrition
Abbreviations: MS, Murashige and Skoog; **NAA**, α-naphthyleneacetic acid

INTRODUCTION

Bananas are popular as a staple food for majority of the tropical population. The crop has great importance for the producers and consumers because of the nutritional and medicinal qualities of the fruits, its production and availability throughout the year, affordable prices and utility of the plant for various industrial purposes. Banana grows well in a temperature range of 18–35°C with a relative humidity regime of 75–85%. Due to the special climatic conditions that are required for optimal growth, the crop is produced mainly in tropical countries and exported to other parts of the world (Shepherd 1987).

Conventional methods of propagation limit crop production due to variation in age and size of the suckers and non-availability of disease free planting material sufficient for planting (Raut and Ranade 2004). As an alternative and efficient method, micro-propagated disease free planting material is being used for several years (Arias 1992). Even though the method is successful, cost of production is high compared to the conventional methods. There are various reports on commercial micro-propagation and low-cost alternatives in banana (Cronauer and Krikorian 1984; Banerjee and de Langhe 1985; Ganapathy *et al*. 1995; Arias 1992; Kodym and Zapata-Arias 2001). Nutrient supply for the cultures causes a major expenditure in the production process.

A comparison of the various studies reported on the *in vitro* multiplication and *ex vitro* conditions and acclimatization of banana (*Musa paradisiaca* L.) variety 'Grand Naine' are presented in **Tables 1** and **2**, respectively. In the present study, the possibilities of reducing mineral salt strength in the root initiation medium of banana were assessed in order to reduce the production cost. Experiments were conducted to standardize the optimal mineral salt concentration for *in vitro* rooting and hardening in variety 'Grand Naine'.

MATERIALS AND METHODS

Explant preparation

Three week-old cultures of banana (*Musa paradisiaca* L var. 'Grand Naine') were selected as source of micro-shoots. Individual shoots were excised aseptically inside a laminar air flow chamber. Two-leaved shoots of about 2.5 cm were selected for the experiment. Shoots (one per test tube) were inoculated in a manner that the corm part was immersed in the medium.

Media preparation

MS basal medium (Murashige and Skoog 1962) was used for root initiation in banana. Four treatment media were prepared (**Table 3**) by decreasing the mineral salt concentration and were designated as full strength (T_1), three-fourth (T_2), half (T_3) and quarter strengths (T_4) respectively. NAA (2 mg l^{-1}) and sucrose (20 g l^{-1}) were added to the media before adjusting the pH to 5.8. Activated charcoal (100 mg l^{-1}) and agar (8 g l^{-1}) were added to each of the treatment media prior to boiling. The media were dispensed into test tubes (15 cm × 2.5 cm) as 10 ml aliquots. The test tubes were closed with poly propylene caps and sterilized by autoclaving at 121°C for 20 min.

Culture conditions

Cultures were incubated at a temperature of 26 ± 2°C with a 16-h photoperiod and a light intensity of 1500 lux. Each replicate consisted of 12–15 shoots and each experiment was repeated three times. Growth response was recorded at weekly intervals.

Hardening

After three weeks of incubation, all the cultures from each treatment group were hardened to assess their performance *ex vitro*. Plantlets were taken out of the test tubes, the agar medium washed off and transferred to egg trays containing sterilized potting mix-

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Table 1 *In vitro* micropropagation studies on banana (*Musa paradisiaca* L.) variety 'Grand Naine'.

Basal medium	Plant growth regulators	Additives	Growth conditions	Results	References
S: MS	S: BAP (5 mg L^{-1})	Sucrose 3%	16 h light, 25 ± 2ºC	9.1 shoots/explant (2-fold increase in Grand Naine compared to other varieties)	Cronauer and Krikorian 1984
S: MS	S: BAP (5-20 mg L^{-1})	Sucrose 10-40%	16 h light, 25 ± 2ºC	10 - 15 mg L^{-1} BAP induced nodule-like meristems	Prioyono 2001
S: MS	S: BAP 17.7 µM	Thiamine HCl (1 mg L^{-1}) + sucrose 3%	Natural light 150 µmol m^{-2} s^{-1}, 27 ± 2ºC	2.4/bud	Albany *et al.* 2005
S: MS	S: BAP 17.7 µM	Thiamine HCl (1 mg L^{-1}) + sucrose 3% + Gellan gum 2 g L^{-1})	Natural light 150 µmol.m^{-2}s-^{1}, 27 ± 2ºC	2.4/bud	Albany *et al.* 2005
S: MS	S: BAP 17.7 µM	Thiamine HCl (1 mg L^{-1}) + sucrose 3% + ANC 9.5 µM	Natural light 150 µmol m^{-2} s^{-1}, 27 ± 2ºC	5.42/bud	Albany *et al.* 2005
S: MS	S: BAP 17.7 µM	Thiamine HCl (1 mg L^{-1}) + sucrose 3% + PBZ (8.5 µm)	Natural light 150 µmol m^{-2} s^{-1}, 27 ± 2ºC	4.56/bud	Albany *et al.* 2005
S: MS	BAP (0, 2, 4 and 6 mg L^{-1})	Activated charcoal (3 g L^{-1})	16 h at 30 µmol m^{-2} s^{-1}, 25 ± 2ºC	Activated charcoal reduced the multiplication rate, improved root number	da Silva Costa *et al*. 2006
S: MS	S: 10 µM (BAP) + 5 µM (IAA)	Sucrose 3%	25 ± 2ºC	3.1 shoots/culture	Ali 2008
S: MS	S: BAP (5 mg L^{-1})	S: Filtered water (Aqua guard®)	25 ± 2ºC	12.5 shoots/culture	Prabhuling *et al.* 2010
S: MS	S: 6 BAP (3 mg L^{-1}) R: IBA (1 mg L^{-1})	S: Adenine sulphate R: charcoal	16-h photoperiod (3000 lux), 25°C	Plants from 8th sub-culture were better in the field than 15th sub-culture	Vishwas *et al* 2010
S: MS R: MS	S: BA (2-5 mg L^{-1}) IAA (1-2 mg L^{-1}) R: IBA (1 mg L^{-1})	Sucrose (20-30 g L^{-1}), agar 0.7%	16 h (3000 lux) R.H.: 40-60%, 25 ± 2ºC	Height :9 cm Leaves: 6 cm Roots: normal	Vasane *et al*. 2010
S: MS	-	S: 30 g L^{-1} sucrose + 9 g L^{-1} agar + pineapple juice 20 ml L^{-1}	25 ± 2ºC	3.66 shoots/culture 6.76 roots/shoot	Beshir *et al*. 2012
S: MS	-	S: 30 g L^{-1} sucrose, and 9 g L^{-1} agar + coconut milk 20 ml L^{-1}	25 ± 2ºC	2.33 shoots/culture 6.06 root/shoot	Beshir *et al*. 2012

ANC, ancymidol; BA, 6-benzyl adenine; BAP, 6-benzylaminopurine; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; MS, Murashige and Skoog basal medium; PBZ, paclobutrazol; S, shooting medium; R, rooting medium; R.H., relative humidity

Table 2 *Ex vitro* conditions and acclimatization studies on banana (*Musa paradisiaca* L) variety 'Grand Naine'.

Medium	Growth conditions	Results	References
P: Organic matter + Zeolite®	P: 25-30°C	ANC treated, 4.92 roots/plantlet	Albany *et al.* 2005
P: Organic matter + Zeolite®	P: 25-30°C	PBZ treated, 5.6 roots/plantlet	Albany *et al* 2005
P: Organic matter + Zeolite®	P: 25-30°C	Control (liquid), 5.7 root/plantlet	Albany *et al.* 2005
P: Organic matter + Zeolite®	P: 25-30°C	Control (semi-solid) 5.65 root/plantlet	Albany *et al.* 2005
P: Cocopith and Soilrite	P: R.H. 100-60%, Temp: 27°C, Days: 45	Survival 91%; No. of roots: 5.7; Root length: 9 cm; No. of leaves: 4.8	Vasane and Kothari 2008
S: Soil	S: R.H: 30-40%, Temp: Ambient 50% light cut off days: 45	Survival 79%; No. of roots: 8.8; Root length: 12.8; No. of leaves: 5.2	Vasane and Kothari 2008
P: Cocopith	P: R.H; 80–90% Temp. 30-35°C Days: 45	8th sub-culture derived plants in the field. Plant height 209.8 cm, No. of leaves 10	Vishwas *et al.* 2010
S: 70% river sand + 30% compost	S: Shade house 60 days	15th sub-culture derived plants in the field. Plant height 203.5 cm, No. of leaves 10	
P: Peatmix	P: R.H: 70%, Temp: 27°C, 15,000 Lux, Days 45	Plantlet height10 cm, No. of leaves 5, Leaf length 8.8 cm, leaf width 4.0 cm	Vasane *et al*. 2010
S: Soil + organic manure	S: 50% shade, Days 45	Survival 100%, plant height 22.9 cm, No. of leaves 8.4, Leaf length 22.6 cm, Leaf width 11.2 cm	Vasane *et al*. 2010

ANC, ancymidol; PBZ, paclobutrazol; P, primary hardening; R.H., relative humidity; S, secondary hardening

ture (1:1:1 ratio of red soil, river sand and farmyard manure sterilized with 4 ml^{-1} formaldehyde). The plantlets in the egg trays were kept inside the mist chamber for further growth and acclimatization.

Data collection and analysis

Weekly observations were made to assess the growth response. Root initiation and development was recorded consecutively for three weeks of *in vitro* phase. Number of leaves, roots per plantlet and the length of the shoot and roots were recorded at the time of hardening. Parameters such as survival rate, number of leaves per plantlet, surface area of the newly developed leaves and shoot length were assessed and recorded for the first two weeks of hardening. After two weeks of preliminary hardening, the plantlets were collected from the egg trays and the number of roots, root length and fresh weight were recorded. The effect of treatment on each parameter was examined using one-way analysis of variance. Individual differences among groups were analyzed employing Tukey's test, $P < 0.05$ was considered as statistically significant (McDonald 2009).

RESULTS

In vitro phase

Root initiation was observed as white protrusions at the base of the cultures of all the treatment groups at the end of the first week. The response of root development during the *in vitro* phase is shown in **Table 4**. The best root initiation response was observed in the treatment group T_2 followed by T_3, T_4 and T_1 groups. However, at the end of the 2nd week, all the cultures (regardless of the treatment) developed roots. The highest number of roots was observed with

Table 3 Composition of treatment media.

Treatment	Strength of MS basal medium	NAA (mg l^{-1})	Sucrose (g l^{-1})	Activated charcoal (mg l^{-1})	Agar (g l^{-1})
T_1	Full	2	20	100	8
T_2	Three-fourths	2	20	100	8
T_3	Half	2	20	100	8
T_4	Quarter	2	20	100	8

Table 4 Root development in banana during the *in vitro* phase.

Treatment	First week	Second week	Third week
	Cultures with roots (%)	No. of roots/culture (Mean ± SEM)	No. of roots/culture (Mean ± SEM)
T_1	82.2	12.91 ± 0.96	22.07 ± 1.39 a
T_2	100	15.42 ± 0.89	27.38 ± 1.82 bc
T_3	93.3	15.93 ± 0.91	21.84 ± 0.99 a
T_4	86.6	13.16 ± 1.01	21.38 ± 1.05 ad

Values in rows sharing the same superscript are not significantly different from each other (ANOVA, Tukey's HSD, $P < 0.05$)

the T_3 group (15.93 ± 0.91) and the lowest in T_1 group cultures (12.91 ± 0.96). At the end of 3rd week, an increase in root number occurred in all the groups (**Figs. 1A-D**). However, this response was significantly high for T_2 group compared to T_1, T_3 and T_4 groups.

Ex vitro phase

At the time of hardening (**Figs. 1E-H**), the plantlets of treatment group T_2 showed a better response in shoot length, root number and root length in comparison to the other treatment groups. However, with respect to leaf numbers, the treatment groups T_2 and T_3, showed a significantly higher values response compared to T_1 and T_4 groups (**Table 5**).

The response in establishment of the plantlets after the first week of hardening in different treatment groups are shown in **Figs. 2A-D**. The highest survival rate was observed in treatment group, T_1 (93.3%) followed by 86.66% in T_2 and T_3 groups and 82.22% in T_4 group. The cultures of all the treatment groups developed new leaves. The treatment group T_1 had a significantly larger leaf area (4.53 ± 0.29 cm^2) compared to the other groups. Shoot elongation was similar for T_1, T_2 and T_3 groups, however, plantlets of T_4 group showed significantly low response (**Table 6**).

The growth response of the plantlets after the second week of hardening in different treatment groups are shown in **Figs. 2E-H**, and also represented in **Tables 7** and **8**. Plantlets of treatment group T_1 had the highest survival rate followed by T_3, T_2 and T_4 groups. Number of plants per group that developed new leaves was more in T_3 group followed by T_1, T_4 and T_2 groups. There were no differences among the groups with respect to the number of new leaves formed. The total number of leaves per plant was highest for T_3 followed by T_2, T_4 and T_1 groups. The leaf area development was more in T_1 and T_2 group compared to T_3 and T_4 groups. The shoot elongation and increase in fresh weight was similar in T_1, T_2 and T_3 groups and these values were significantly higher than that of the T_4 group. Root elongation was higher for T_1 and T_2 groups compared to the T_3 and T_4 groups. However, there were no statistical differences between the values for the T_2, T_3 and T_4 groups. Plantlets of T_4 group had low survival rate, leaf area, mean shoot length and fresh weight compared the other groups.

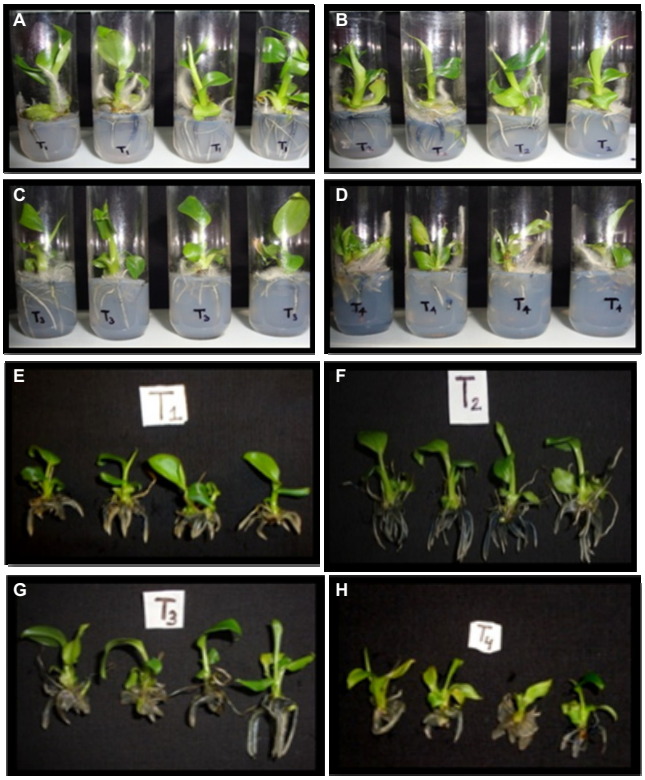

Fig. 1 Growth response of banana cultures at the end of *in vitro* phase prior to *ex vitro* hardening. A, **B**, **C**, **D** - cultures in T_1, T_2, T_3 and T_4 media; **E**, **F**, **G**, **H -** plantlets from T_1, T_2, T_3 and T_4 media.

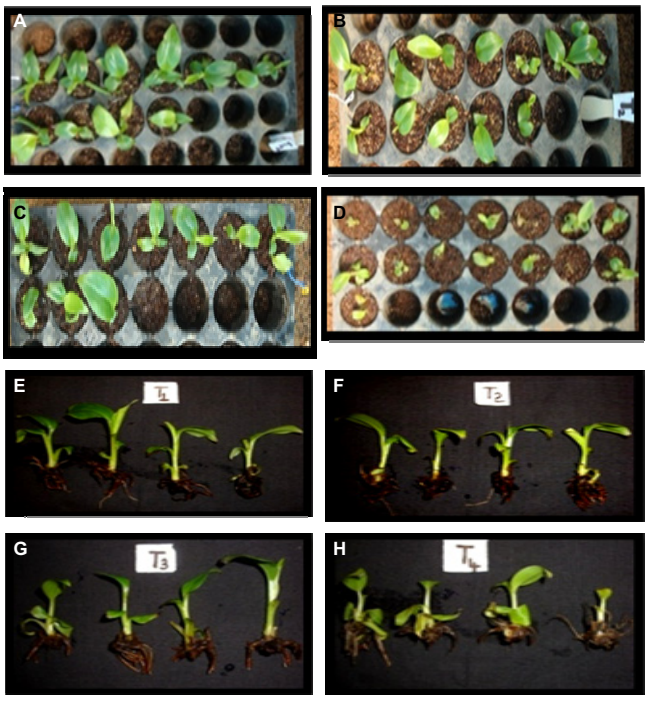

Fig. 2 Plantlets of banana at the *ex vitro* hardening phase. A, **B**, **C**, **D** - one week-old plantlets of T_1, T_2, T_3 and T_4 groups; **E**, **F**, **G**, **H** - two week-old plantlets of T_1 , T_2, T_3 and T_4 groups.

DISCUSSION

Routine procedure for *in vitro* plantlet production and hardening involves rooting of micro- shoots on media supplemented with full strength mineral salt mixture. Even though micro propagated banana plants have many advantages, laborious processes and high production costs remain as limitations for the large scale production of propagules. The present study was designed to find out a commercially viable *in vitro* rooting and hardening procedure. For this purpose, the mineral salt concentrations in the root initiation media were reduced from full-strength (T_1) to three-fourth (T_2), half- (T_3) and quarter- (T_4) strength and the effect of low mineral salt strength at various stages of plantlet development was assessed.

The treatment group T_2 with three-fourth strength basal media induced roots at a significantly high ratio compared

Table 5 Growth response of banana cultures at the hardening phase.

Treatment	No. of leaves (Mean ± SEM)	Shoot length (Mean ± SEM)	No. of roots (Mean ± SEM)	Root length (Mean ± SEM)
T_1	3.40 ± 0.14 a	2.18 ± 0.08 ac	22.07 ± 1.39 a	1.81 ± 0.08 a
T_2	3.58 ± 0.17 ab	2.29 ± 0.14 ab	27.38 ± 1.82 b	1.94 ± 0.10 a
T_3	4.00 ± 0.12 bc	1.88 ± 0.10 c	21.84 ± 0.99 ac	1.50 ± 0.06 bc
T_4	3.22 ± 0.18 a	0.98 ± 0.06 d	21.38 ± 1.05 a	1.49 ± 0.06 c

Values in rows sharing the same superscript are not significantly different from each other (ANOVA, Tukey's HSD, $P < 0.05$)

Table 6 Response of banana plantlets at the end of first week of hardening.

Treatment	Survival (%)	Plantlets with new leaves (%)	No. of new leaves (Mean ± SEM)	Leaf area (cm^2) (Mean ± SEM)	Shoot length (cm) (Mean ± SEM)
T_1	93.30	100	1.24 ± 0.06	4.53 ± 0.29 a	1.47 ± 0.08 a
T_2	86.66	100	1.41 ± 0.09	2.95 ± 0.24 b	1.36 ± 0.09 ab
T_3	86.66	100	1.41 ± 0.08	2.85 ± 0.18 b	1.39 ± 0.06 ab
T_4	82.22	100	1.48 ± 0.12	0.87 ± 0.11 c	0.69 ± 0.06 c

Values in rows sharing the same superscript are not significantly different from each other (ANOVA, Tukey's HSD, $P < 0.05$)

Table 7 Leaf development of plantlets at the end of second week of hardening.

Treatment	Plants with new leaves (%)	Total no. of leaves (Mean ± SEM)	Leaf area (cm^2) (Mean ± SEM)
T_1	36.84	4.92 ± 0.215 a	3.29 ± 0.41 a
T_2	30.55	5.28 ± 0.23 ab	2.46 ± 0.45 ab
T_3	37.83	5.84 ± 0.15 bc	2.00 ± 0.29 b
T_4	31.42	5.08 ± 0.23 ac	0.63 ± 0.16 c

Values in rows sharing the same superscript are not significantly different from each other (ANOVA, Tukey's HSD, $P < 0.05$)

Table 8 Shoot development of banana plantlets at the end of second week of hardening.

Treatment	Plantlet survival (%)	Shoot length (Mean ± SEM)	Root length (Mean ± SEM)	Fresh weight (g)
T_1	84.44	2.88 ± 0.09 a	3.20 ± 0.13 a	1.12 ± 0.07 a
T_2	80	2.67 ± 0.11 a	2.78 ± 0.09 abc	1.15 ± 0.06 a
T_3	82.22	2.88 ± 0.11 a	2.66 ± 0.13 b	1.09 ± 0.04 a
T_4	77.77	1.60 ± 0.10 b	2.66 ± 0.16 bc	0.81 ± 0.03 b

Values in rows sharing the same superscript are not significantly different from each other (ANOVA, Tukey's HSD, $P < 0.05$)

to the rest of the treatments and all the cultures responded within first week itself. The routine medium, T_1 (full-strength) showed a comparatively low response. Full-strength and quarter- strength mineral supplementation in the rooting media delayed root initiation in the crop. Three-fourth and half-strength mineral concentrations were similar their *in vitro* and *ex vitro* responses. These findings indicate that the concentration of minerals is critical for *in vitro* root development as well as their performance *ex vitro*. The plantlets grown on three-fourth and half-strength media showed similar responses for leaf number, shoot length, root number and root length. However, at the time of transfer from *in vitro* to *ex vitro* condition, three-fourth strength was determined as optimal for root development. In a study on e*x vitro* rooting of micro-shoots of banana var. 'Grand Naine', Thirugnanasambandam (2008) recorded that half-strength mineral salt media was the optimal nutrient supplement for *ex vitro* root development and acclimatization of plantlets.

During the first week of hardening, plantlets grown on three-fourth and half-strength showed similar response for all the parameters compared to full and quarter-strength treatment groups. During the second week of hardening, T_2 and T_3 groups showed very good performance with respect to survival rate, leaf development, total number of leaves and shoot elongation. Additionally, increase in fresh weight was observed for the plantlets of the T_2 group. However, there were no significant differences between the T_2 and T_3 groups.

The response obtained from the *in vitro* and *ex vitro* studies indicates that slightly low concentration of mineral supplements was efficient for *in vitro* rooting and hardening in the variety 'Grand Naine'. Improved performance in the presence of low concentration of mineral supplements was reported by Ganapathy *et al*. (1995). These authors studied the effect of mineral supplementation in liquid media by using MS medium and Knop's solution for the rooting process. Knop's solution, which had low mineral concentration, was efficient for rooting in the variety 'Basrai'. Low mineral supplements might have enhanced photoautotrophic responses of the plantlets. It has also been reported that successful hardening and acclimatization depends on the photoautotrophic development of the plantlets *in vitro* (Kozai 1991; Ganapathy *et al*. 1995; Kodym and Zapta-Arias 1999, 2001). The results from the present study were in accordance with the above reports.

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