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Microbial hardening of tissue-culture raised Matti banana (*Musa acuminata*) plants regenerated from immature male flowers

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Abstract
The establishment of micropropagated banana plantlets is challenging in field conditions and in order to improve its sturdiness, the inoculation of beneficial microbes is carried out in the Matti banana plants regenerated *in vitro* using the immature male flowers as explant. In the present investigation, morphological, biochemical, nutritional and mineral content status of tissue-cultured Matti banana plantlets in response to nine treatments of beneficial microbial inoculants was studied. The results revealed that the treatment comprising of *A. brasilense*, *B. megaterium*, *B. amyloliquefaciens* and VAM mixed culture each 1 g in quantity was superior in enhancing the growth with respect to plant height, pseudostem girth and the number of leaves. The biochemical studies revealed higher chlorophyll, carotenoid and flavonoid content compared to the uninoculated control plants. With respect to nutritional status for carbohydrate, protein and fat content and also mineral composition for nitrogen, phosphorus and potassium percentage also, there was significant accumulation. These results were indicative of better acclimatization or hardening of micro-propagated Matti banana plantlets through microbial inoculants, the reason being attributed to microbial rhizosphere providing protection from pathogenic organisms and enhanced nutrient uptake.

Keywords: Matti banana, micropropagation, microbial inoculants, hardening, growth, biochemical, nutritional, minerals

Introduction
Banana is the most important crop in India (Anon, 1999) [3] and micropropagation by shoot tip culture is used for commercial production of plantlets. Since shoot tip culture poses problem of contamination in the cultures, immature male flowers have been used for multiplication. Hardening or acclimatization is a very important aspect in the case of tissue-cultured plants since *in vitro* developed plants are much prone to shock during transfer to soil-based substrate. Even though nutrient solutions are poured to the substrate, addition of beneficial microbes will be more applicable since the substrate is sterilized for transferring the *in vitro* grown plants into it. Successful hardening process is very much essential for increasing the survival rate of in vitro grown plants (Radheshyam and Subramani, 2008) [25]. Bacteria and mycorrhizal fungi by forming a colony surrounding the rhizosphere, release nutrients near the root system and also protect the plants from pathogenic microbes (Weller *et al*., 2002 and Fravel *et al*, 2003) [34, 9]. Arbuscular mycorrhizal colonization of plants stimulates growth of plants by enhancing nutrients uptake and growth hormone production (Gerdemann, 1968) [10]. The plantlets developed by tissue culture have divergent physiology (Pierik, 1987) [28] and to improve the sturdiness of the *in vitro* plants, application of bioinoculants will be beneficial. Hence it is essential to study the influence of bioinoculants on the biological processes that play a role in acclimatization of tissue-cultured Matti banana plants regenerated from immature male flower explants.

Materials and Methods
Micro-propagated banana plantlets
Tissue-cultured Matti banana plants were produced by culturing its immature male flowers as explants. The male inflorescence was collected from healthy mother plants and washed in tap water. In the Laminar Air Flow chamber, the bracts were removed up to the size of 10cm and the bud was sterilized using 6% sodium hypochlorite for 10 minutes. The buds were thoroughly washed using sterile distilled water and dissected to excise the male flower clusters at 12th to 15th position of the inflorescence.

The clusters were then inoculated in the MS medium with BAP 2 mg/lit and kinetin 1 mg/lit for initiation and BAP 4 mg/lit for multiple shoot formation (Fig 1). Once in four weeks, the micro-shoots were sub-cultured in MS medium with BAP 5 mg/lit for six cycles (Fig 2). The shoots formed were transferred to rooting media of half MS with IBA 1 mg/l. The plantlets regenerated were used for primary and secondary hardening by various treatments in polyhouse and shade net house respectively (Fig 3).

Microbial inoculants

The substrate used for hardening was a mixture of soil, FYM and cocopeat in 1:1:1 ratio sterilized and inoculated with microbial cultures *viz.*, nitrogen fixer (NF), *Azospirillum brasilense* ($1x10^9$/g), phosphobacteria (PB), *Bacillus megaterium* var. *phosphaticum* ($1x10^8$/g), potash solubilising bacteria (KSB), *Bacillus amyloliquefaciens* ($1x10^8$/g), vesicular arbuscular mycorrhizae (VAM) mixed culture comprising of *Glomus* sp, *Gigaspora* sp, *Acaulospora* sp and *Sclerocystis* sp (30 spores/g inoculam) and biocontrol agent (BCA), *Bacillus subtilis* ($1x10^8$/g) obtained from AC&RI, Killikulam. These cultures were added to the substrate at various combinations and the treatments are as follows.

Microbial inoculants

Treatments	Microbial inoculants (g)				
	NF	PB	PSB	VAM	BCA
T_1	0.5	0.5	0.5	0.5	0.5
T_2	0.5	0.5	0.5	0.5	-
T_3	0.5	0.5	0.5	-	0.5
T_4	0.5	0.5	0.5	-	-
T_5	1.0	1.0	1.0	1.0	1.0
T_6	1.0	1.0	1.0	1.0	-
T_7	1.0	1.0	1.0	-	1.0
T_8	1.0	1.0	1.0	-	-
T_9	Control - uninoculated				

After preparation of the substrate with various combination of microbial inoculants, the portrays and polybags were filled with the substrate with all the treatments and *in vitro* Matti banana plantlets were planted for primary hardening in poly house and for secondary hardening in shade net house respectively. The observations were taken on the 45th day of primary hardening (PH) and secondary hardening (SH).

Morphological parameters

Plant height in centimetres was measured from the base of the plant to the shoot tip. The pseudostem girth in centimetres was measured at the base of the plant and for the number of leaves, the fully opened leaves were counted.

Biochemical parameters

The amount of chlorophyll and carotenoids were estimated spectrophotometrically and flavonoid content was determined following the method of Park *et al.*, 2008 [27].

Nutritional parameters

After primary and secondary hardening, the total carbohydrates, protein and fat were estimated. The total carbohydrates content was measured by Anthrone reagent (Hedge and Hofreiter, 1962) [11]. The method developed by Lowry *et al.*, 1951 [20] was used to determine the protein content. The Soxhlet method was adopted for the calculation of total fat content (AOAC, 1995; Strugnell, 1989) [4, 32].

Mineral content estimation

Total nitrogen was estimated by micro-Kjeldahl method as per the procedure suggested by AOAC, 1995 [4]. The calorimetric determination of phosphorus in the plant sample was measured by Vanado-molybdate method (Olsens *et al.*, 1954) [23]. The estimation of potassium is carried out by flame photometer (Black, 1965) [8].

Completely randomized design was followed for the experiment. Three replications were kept for all the nine treatments. The data was analysed statistically and tested for significance using AGRES software.

Results and Discussion

The inoculation of microbial agents enhanced the physical status of the micropropagated banana, Matti with respect to plant height. The treatment T6, constituting of *A.brasilense*, *B. megaterium*, *B. amyloliquefaciens* and VAM mixed culture each 1g in quantity was superior in enhancing the plant height both in primary (15.6cm) and secondary (30.5cm) hardening process. Also, the plantlet girth was 0.82cm and 1.25cm and the number of leaves recorded was 2.5 and 5.8 in respective hardening processes (Table 1 & Fig 4). The finding is in accordance with the other results (Singh and Chundawat, 2002; Shailesh *et al.*, 2006, Jie *et al.*,2009, Lian *et al.*,2009, Kavino *et al*, 2010, Anbazhagan *et al.*, 2014, Mon *et al.*, 2021) [30, 29, 19, 14, 2, 22].

The biochemical components were higher in treatment T6 with chlorophyll content of 1.14 mg/g and 1.5 mg/g, carotenoid content of 0.152 mg/g and 0.178 mg/g and flavonoid content of 7.9 mg/g and 9.78 mg/g respectively in primary and secondary hardening (Table 2). The results are in accordance with the reports of Krishna *et al.*, 2005 [17], Van Loon, 2007 [33] and Beneduzi *et al.*, 2012 [7]; Ramesh and Ramasamy, 2015 [26].

The nutritional status of the plantlets when analysed showed that the carbohydrates content was 30.32% and 39.17%, the protein content was 5.45% and 6.72% and the fat content was 1.01% and 1.185 respectively in primary and secondary hardening stages (Table 3). Indravathi and Suresh Babu, 2019 [16] has reviewed the nutritional aspects of tissue cultured plants due to biotization.

The mineral content when studied recorded 22.5 mg/g and 24.9 mg/g for nitrogen, 1.55 mg/g and 1.72 mg/g in the case of phosphorus and 30.1 mg/g and 32.9 mg/g for potassium respectively in primary and secondary hardening processes (Table 4). Microorganisms act as a promising factor in improving the soil fertility and increase the nutrient absorption (Akhtar *et al.*, 2012; Posada *et al.*, 2016; Kumar *et al.*, 2020) [1, 24, 18].

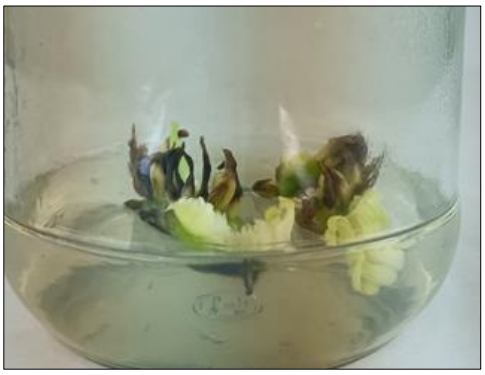

Fig 1: Initiation of immature male flowers of Matti banana

Table 1: Effect of inoculation of microbial inoculants on the morphological parameters of micropropagated Matti banana.

Treatments	Plant height (cm)		Pseudostem girth (cm)		No of leaves/ plant	
	PH	SH	PH	SH	PH	SH
T_1	13.4	24.8	0.63	1.12	2.1	4.2
T_2	13.2	25.3	0.68	1.17	2.2	4.3
T_3	12.5	23.4	0.61	1.10	2.1	4.0
T_4	11.0	22.0	0.58	0.97	2.0	3.8
T_5	15.0	30.1	0.79	1.22	2.4	5.5
T_6	15.6	30.5	0.82	1.25	2.5	5.8
T_7	13.4	28.2	0.75	1.20	2.3	5.0
T_8	12.3	25.0	0.67	1.16	2.1	4.8
T_9	9.0	18.6	0.45	0.71	2.0	3.0
CD at 5%	0.66	1.19	0.037	0.054	0.05	0.28

Table 2: Effect of inoculation of microbial inoculants on the biochemical parameters of micropropagated Matti banana.

Treatments	Chlorophyll (mg/g)		Carotenoids (mg/g)		Flavonoid (mg/g)	
	PH	SH	PH	SH	PH	SH
T_1	1.09	1.41	0.139	0.165	6.85	8.71
T_2	1.10	1.38	0.134	0.161	6.81	8.66
T_3	1.07	1.35	0.121	0.153	6.13	8.11
T_4	1.03	1.28	0.112	0.136	5.95	6.80
T_5	1.11	1.50	0.147	0.170	7.84	9.35
T_6	1.14	1.53	0.152	0.178	7.90	9.78
T_7	1.08	1.40	0.126	0.157	6.92	8.60
T_8	1.05	1.31	0.115	0.145	6.22	7.97
T_9	0.96	1.15	0.102	0.114	3.89	5.12
CD at 5%	0.02	0.04	0.007	0.009	0.25	0.47

Table 3: Effect of inoculation of microbial inoculants on the nutritional parameters of micropropagated Matti banana.

Treatments	Carbohydrate (%)		Protein (%)		Fat (%)	
	PH	SH	PH	SH	PH	SH
T_1	28.82	36.50	5.01	5.83	0.85	1.03
T_2	27.85	35.97	4.89	5.84	0.80	1.05
T_3	26.51	33.84	4.03	4.79	0.77	0.92
T_4	24.60	32.60	3.52	4.02	0.71	0.76
T_5	30.71	38.96	5.43	6.75	0.97	1.14
T_6	30.32	39.17	5.45	6.72	1.01	1.18
T_7	28.90	35.72	4.75	5.80	0.83	1.02
T_8	26.33	33.57	3.97	4.91	0.74	0.89
T_9	22.05	29.81	2.30	3.52	0.37	0.48
CD at 5%	0.57	0.74	0.21	0.36	0.05	0.06

Table 4: Effect of inoculation of microbial inoculants on the mineral content of micropropagated Matti banana.

Treatments	Nitrogen (mg/g)		Phosphorus (mg/g)		Potassium(mg/g)	
	PH	SH	PH		PH	SH
T_1	21.1	22.7	1.12	1.23	28.1	30.6
T_2	20.9	22.1	1.14	1.20	27.9	30.1
T_3	19.5	21.3	1.05	1.11	26.2	28.5
T_4	18.4	20.5	0.91	0.98	24.4	25.6
T_5	22.0	24.5	1.49	1.69	29.8	32.1
T_6	22.5	24.9	1.55	1.72	30.1	32.9
T_7	20.7	22.9	1.15	1.20	27.7	29.8
T_8	19.3	21.2	1.07	1.09	25.8	27.7
T_9	15.2	18.6	0.68	0.81	17.6	20.8
CD at 5%	0.63	0.45	0.87	0.05	0.52	0.91

Fig 2: Microshoots developed *in vitro*

Fig 3: Secondary hardening in shadenet house

T_1 T_2 T_3 T_4 T_5 T_6 T_7 T_8 T_9

Fig 4: Growth of secondary hardened Matti banana plantlets in various treatments of microbial inoculants.

Conclusion

Based on this study, the microbial inoculants are recommended as efficient component for improving the quality of micropropagated Matti banana during hardening. In addition, the biohardened plants showed good field establishment, disease control and increased productivity (Mia *et al*., 2005; Kavino *et al*., 2007; Soumare *et al*., 2021) [15, 31]. Hence this study has proved that the treatment of micropropagated banana with bioinoculants promote the physical and biological status of *in vitro* Matti banana in the hardening stage.

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