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IN VITRO MULTIPLICATION OF LOCAL CULTIVARS OF BANANA (*MUSA* spp.) THROUGH SHOOT-TIP CULTURE

K. HIRIMBUREGAMA* and N. GAMAGE
Department of Botany, University of Colombo, P.O. Box 1490, Colombo 03.

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Abstract: *In vitro* shoot-tip culture is a suitable alternative to the traditional methods of propagation of banana (*Musa* spp). In the present study, ten banana cultivars of the group AAA, AAB and ABB were tested for *in vitro* multiplication. The study revealed that shoot-tip culture technique can be used for mass propagation of the local cultivars of banana. A variation in multiplication rate was seen not only among different genomic groups but also among cultivars of the same group: highest multiplication was observed in Binkehel (AAA) while the lowest was in Alukehel (ABB) and Suwandel (AAB). Thus, multiplication rate appears to be cultivar dependant. The study also showed that subculturing enhances shoot multiplication, especially after the second subculture.

Key words: *Musa*, banana, micropropagation, shoot-tip culture.

INTRODUCTION

Banana (*Musa* spp.) is a popular fruit in Sri Lanka. Naturally occurring banana hybrids are grouped according to their genome constitutions, as AA, AAA, AB, AAB, ABB and ABBB[1,2] (genome 'A' from *M. acuminata* and the genome 'B' from *M. balbisiana*). These include 300-500 cultivars spread throughout the world. Tetraploid AAAA cultivars have only been produced through breeding programmes.[3] Up to now triploid and tetraploid B cultivars have not been identified.[1,2,4,5] Twenty nine banana cultivars, including cooking and dessert types, have been reported in Sri Lanka.[6]

Until recently, banana cultivation was restricted to home gardens and to small plots of land. With increasing local demand and potential for export markets, large scale cultivation of banana has become common, especially in Gampaha, Uda Walawe, Embilipitiya, Moneragala and Rambukkana. Since almost all the edible bananas are triploid and seed sterile, suckers have been the traditional planting material. Their poor availability is a constraint for commercial cultivation. *In vitro* shoot-tip culture of banana allows rapid clonal multiplication. The technique for the establishment of banana plants in large quantities from excised shoot tips was first reported by Ma and Shii[7] and had been later modified by others.[8-11] This technique is successfully used for mass propagation of banana in some countries including China, India, Thailand and Malaysia. In Sri Lanka, this technique had been tested for some cultivars of banana by the Agriculture Department. However, details of the rates of multiplication of local cultivars are not available. The present study reports on the multiplication rates of some important local cultivars of banana through the shoot tip culture technique.

* Corresponding author.

METHODS AND MATERIALS

Explant: The shoot tip (meristem and a few leaf primordia) was the starting material (Fig. 1 a & b). Healthy sword suckers were used to excise shoot apices.[11]

Cultivars of banana tested: The following local cultivars were tested (genomic constitution is given within parentheses):

* Embul (AAB)
* Suwandel (AAB)
* Puwalu (AAB)
* Kolikuttu (AAB)
* Rathkehel (AAB)
* Binkehel (AAA)
* Pisang Embon (AAA)
* Mondan (ABB)
* Seenikehel (ABB)
* Alukehel (ABB)

Surface sterilization: Suckers were initially washed to remove the adherent soil. A few leaf sheaths close to the base were removed and washed again. The shoot apex area was isolated by 4-6 angular cuts in the parent corm. The final size of the explant was about 2-3 cm in length and about 2.5 cm in diameter. The cut pieces were washed well with soap (Sunlight™) and were surface sterilized by immersing in ethanol (70% v/v, 96% commercial grade) for 10-15 min followed by 20-30 min in Clorox (a commercial bleach, 5.25% NaOCl). Cut pieces were swirled occasionally in each solution. Under sterile conditions, the leaf bases of the cut stem piece were removed and the white shoot tip (meristem with 2-3 leaf primordia) was isolated (Fig. 1 b). Each shoot apex was cut longitudinally into two and each piece was placed on a culture medium (given below) in an upright position (each tube was inoculated with one half of the shoot tip).

Establishment of proliferating shoots: Culture medium: For shoot multiplication, the medium consisted of Murashige and Skoog (1962)[12] basic mineral components with benzyl amino purine (BAP, 2.5 mg/L) and indole acetic acid (IAA, 1.25 mg/L).[10] After adjusting the pH of the medium to 5.8 ± 0.1 with 1M NaOH or 1M HCl, an aliquot of 15 ml of the medium was dispensed into tubes (150 mm length and 24 mm diameter, pyrex). The stoppered tubes with the medium were autoclaved at 1.2 KPa and 121°C for 20 min. Inoculated tubes were incubated under continuous irradiance of 4.2 W/m^2 provided by Osram cool white fluorescent tubes at a temperature of 28 ± 1°C and at 80% relative humidity.

Browning of the explant: Browning was observed in some cultivars within 4-5d after inoculation of the explant. To avoid browning, after about one week, the brown tissues on the explant were removed and the tip was transferred to a similar fresh medium. This was repeated every week for about 3wks to minimize further browning of the tissues.

Maintenance of proliferating shoots : Initial subculturing was done when the explant (shoot-tip) had produced 6-8 shoots. For subculturing, the entire sample of shoots *in vitro* were cut into 2-3 pieces so that each piece would contain about 3 shoots.Leaves if present, and the browned basal tissues were removed to expose the meristems. Each piece was inoculated onto a similar fresh medium.

Cultures were maintained by subculturing in the above manner, every 5-6wks. Through subculturing, the number of shoots were increased.

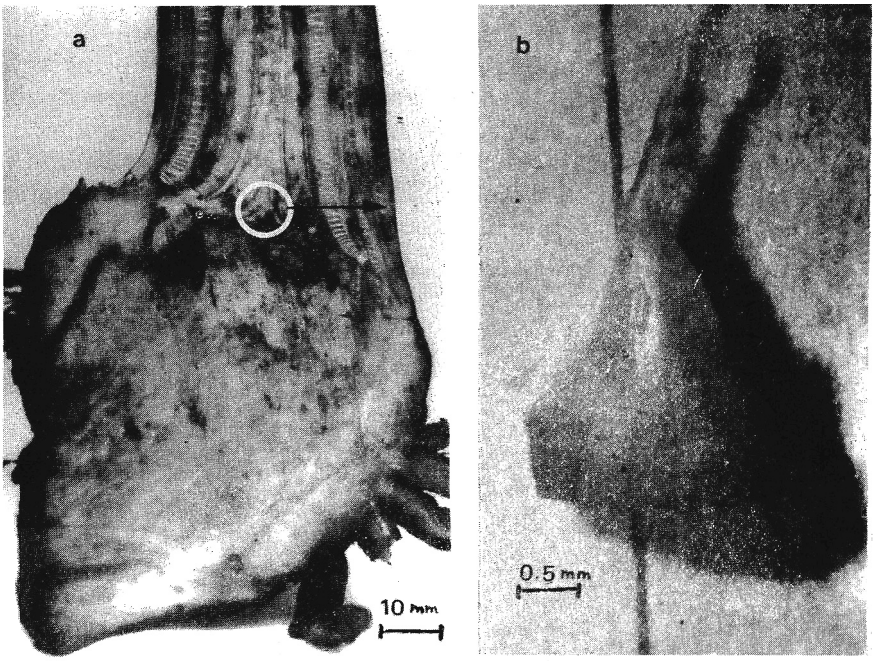

Figure 1: The explant: shoot tip of *Musa*: (a) LS of a banana rhizome to illustrate the position (encircled) of the shoot tip, (b) an isolated shoot tip.

Evaluation of multiplication rate: Every week after inoculation of the explant, the number of shoot-tips was reported for each cultivar. Each cultivar had 15 replicates and the rate of multiplication was tested in five successive subcultures over a period of about 6 months. Comparisons between subcultures of each cultivar and between cultivars were made by using Tukey's HSD test at 5% level of significance.

Regeneration of plants from in vitro proliferated buds: Proliferated meristems *(in vitro)* were separated and each shoot meristem was placed on a culture medium for shoot development. The culture medium consisted of Murashige and Skoog (MS)[12] basic medium supplemented with BAP (1.25 mg/L) and IAA (1.25 mg/L) (at reduced level of BAP). 25 shoots from each cultivar were tested for plant regeneration. For root development, the shoots were placed on basic MS with indole butyric acid (IBA, 1.25 mg/L) for 2wks. Once complete plants were developed they were transferred to pots containing sand:soil (1:1) for acclimatization in the glasshouse.

RESULTS

Multiplication of shoot meristem

In general, from about 7-8wks after inoculation, shoot-tip proliferation could be seen. The meristem of the explant (shoot-tip) multiplied producing several meristems/shoots (Fig. 2 a & b). Each can be considered as a bud due to its ability to develop into a plant. Multiplication of shoot meristem (shoot proliferation) was observed in all the cultivars tested (Table 1). However, there was a difference in the time taken for initiation of multiplication and in the rate. Also, there was a difference in multiplication rate in subcultures of the same cultivar.

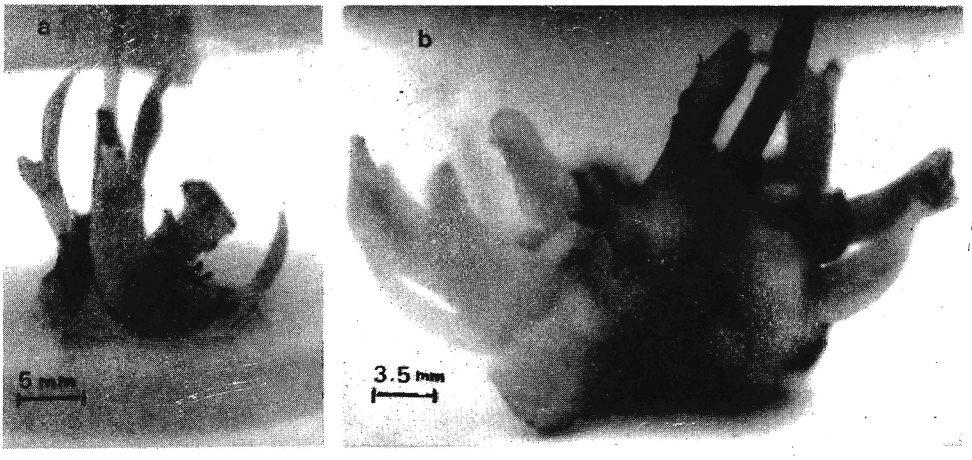

Figure 2: Proliferation of shoots in *Musa*: (a) an early stage, (b) just before subculture.

Binkehel and Pisang Embon - (AAA)

In Binkehel, proliferation of shoot meristems started 6 weeks after inoculation. The number of shoots produced per culture was less than 20 up to seventeen weeks. Therefore, the first subculture was done at week 12, when the mean number of shoots was 8 (Fig. 3a). After third subculture at week 17, there was a significant increase in shoot proliferation (Fig. 4a and Table 1). Pisang Embon, which is also AAA, showed a comparatively lower number of shoots with time (Fig. 3a). First subculture was done when there were 6 shoots at week 13. Thereafter, an insignificant increase was observed in shoot multiplication up to 18 weeks when the second subculture was done (Fig. 4a). However, there was an increase in shoot multiplication with subculturing (Fig. 4b). This increase was significant from the second subculture (Table 1).

Embul, Suwandel, Puwalu, Kolikuttu, and Rathkehel (AAB)

In cultivars Embul, Suwandel, Puwalu and Kolikuttu shoot proliferation started at 7-8wks after inoculation. However in Rathkehel, it was delayed until 17 weeks (Fig. 3b). First subculture in Embul, Suwandel, Puwalu and Kolikuttu

was done at week 13-15 when the number of shoots was 6-8. However in Rathkehel first subculturing was done at week 16 even though the number was 4. This was to study the effect of subculturing on shoot proliferation.

In Embul, there was a comparatively rapid increase in shoot proliferation especially from the second subculture (Fig. 4f). The increase in subsequent subcultures was significant (Table 1). However, in Suwandel and Rathkehel, the multiplication was comparatively less (Fig. 4i & 4j) but there was a significant increase in the number of shoots produced between several subcultures (Table 1).

Alukehel, Mondan and Seenikehel (ABB)

In all the above cultivars of ABB, shoot proliferation started around 8-9 weeks after inoculation. First subculturing in Seenikehel and Alukehel was done at 14 and 15 weeks respectively. In Mondan it was done at week 17 due to slow development of shoots. In Seenikehel, after second subculture at week 19, there was a gradual increase in shoot proliferation (Fig. 3c & 4e) and the increase in subsequent subcultures was significant (Table 1). However in Mondan and Alukehel, the proliferation rate was comparatively less and was similar to one another (Fig. 3c, 4c & 4d).

Rate of shoot proliferation with subcultures

In all 10 cultivars tested, the total number of shoots increased with subculturing. In most cultivars, the increase in the number of shoots was significant after the first subculture. Multiplication was relatively high in Binkehel, Pisang Embon and Puwalu but was poor in Alukehel, Rathkehel, Suwandel and Mondan.

According to the results, the increase was most prominent in Binkehel in which the total number of shoots after the 5th subculture was more than 70. In Embul, it was 66. However, in Rathkehel, Suwandel, Alukehel and Mondan,the number was below 25.

Browning of tissues and contamination

Browning was one of the difficulties encountered, especially at the initiation of the shoot tip cultures. Comparatively, a high amount of browning was observed with ABB and AAB cultivars. Several transfers had to be done to minimize browning.

Contamination of cultures in general was around 3-5%.

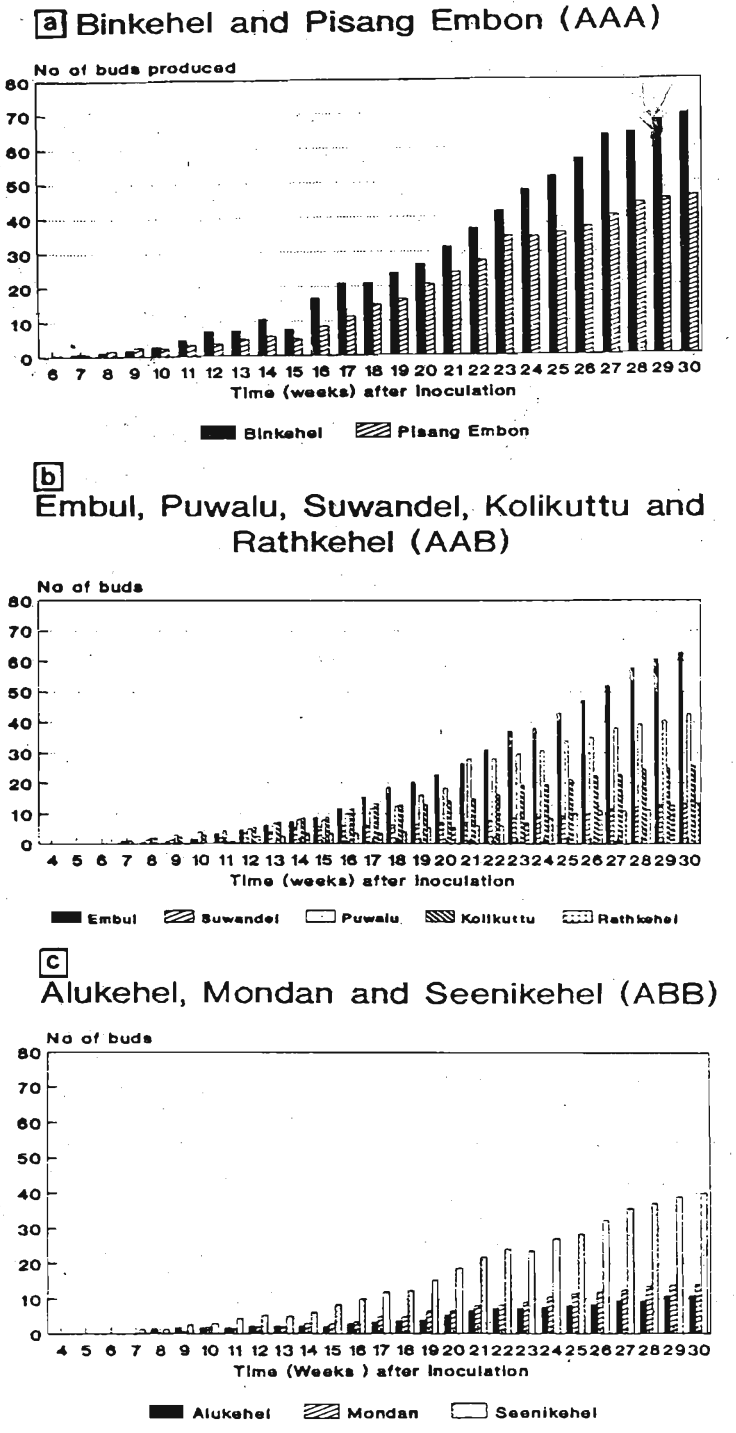

Figure 3: Shoot multiplication in the tested cultivars.

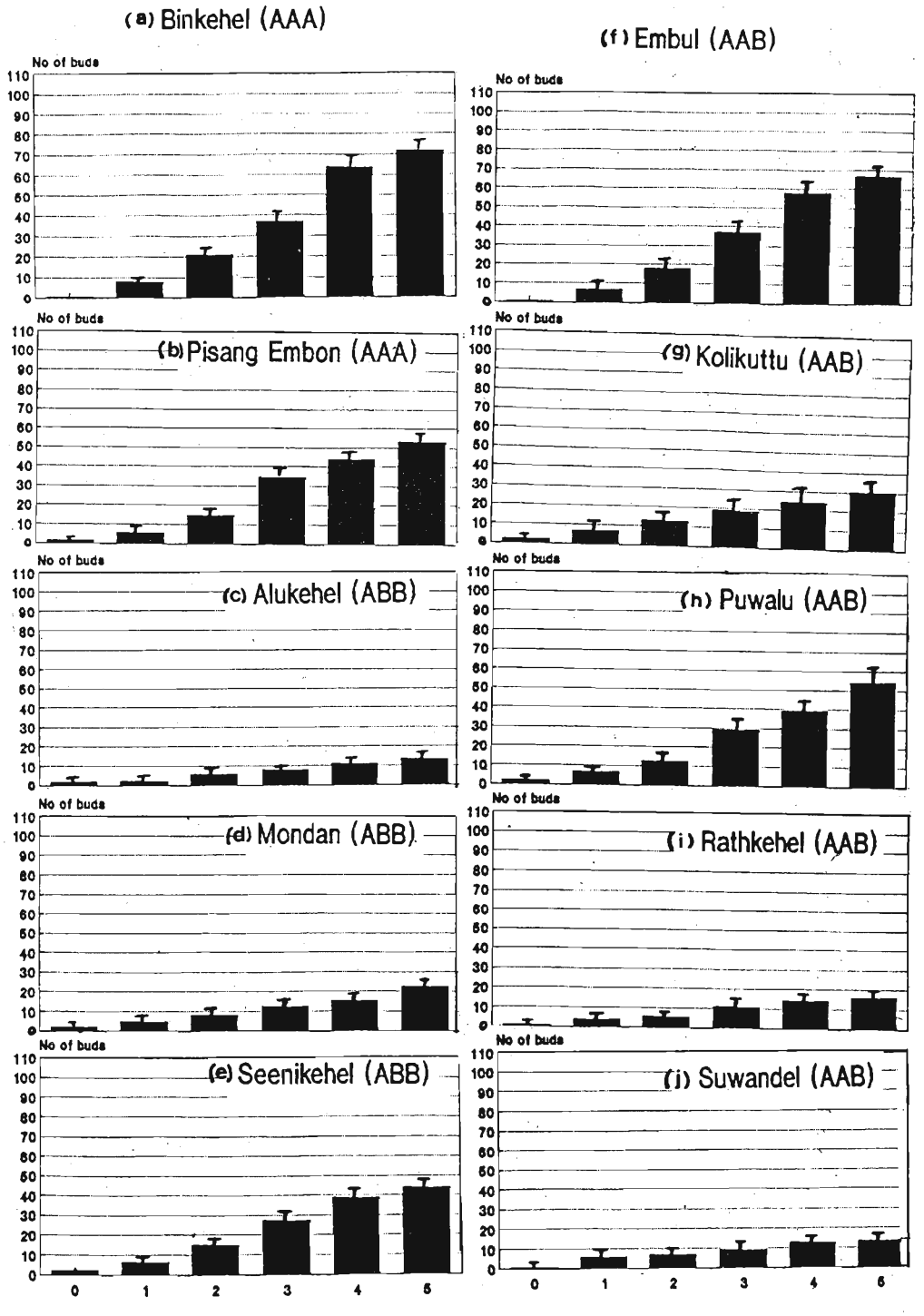

Figure 4: The number of buds produced at each subculture by the tested banana cultivars. (0,1,2,3,4,5 are the initial culture and from first to fifth subculture)

Table 1: The number of shoots produced in each subculture (starting from a single shoot-tip, over a total period of 6-8 months).

Cultivar	Subculture Number					
	S_0	S_1	S_2	S_3	S_4	S_5
Binkehel (AAA)	0.7 ± 0.3^{a}	7.7 ± 0.6^{ab}	21.1 ± 0.6^{bc}	36.9 ± 5.1^{c}	63.7 ± 6.3^{de}	72.1 ± 5.0^{e}
Pisang Embon (AAA)	1.8 ± 0.2^{a}	5.8 ± 1.1^{a}	14.8 ± 3.0^{b}	34.6 ± 2.5^{c}	44.4 ± 2.4^{d}	53 ± 1.5^{e}
Embul (AAB)	0.5 ± 0.2^{a}	6.6 ± 0.5^{a}	18.0 ± 2.4^{b}	37.3 ± 3.8^{c}	58 ± 2.1^{de}	66.5 ± 1.8^{e}
Puwalu (AAB)	2.2 ± 0.4^{a}	6.6 ± 0.3^{b}	12.4 ± 0.4^{c}	29.2 ± 0.7^{d}	39.4 ± 1.0^{e}	54.2 ± 0.9^{f}
Suwandel (AAB)	0.5 ± 0.3^{a}	5.8 ± 0.6^{bc}	7.0 ± 0.4^{cd}	9.3 ± 0.3^{d}	13 ± 0.9^{ef}	13.5 ± 0.9^{f}
Kolikuttu (AAB)	2.2 ± 0.2^{a}	7.2 ± 0.4^{b}	12.8 ± 0.2^{c}	19.2 ± 0.9^{d}	24.6 ± 0.9^{e}	30.2 ± 1.3^{f}
Rathkehel (AAB)	0.8 ± 0.5^{a}	3.8 ± 0.3^{ab}	5.5 ± 0.9^{b}	11 ± 0.9^{cd}	14.3 ± 0.9^{de}	16.3 ± 0.9^{e}
Alukehel (ABB)	2.0 ± 0^{a}	2.5 ± 0.3^{a}	5.5 ± 0.3^{b}	8.0 ± 0.6^{cb}	11 ± 0.9^{d}	13.8 ± 0.9^{e}
Mondan (ABB)	2.0 ± 0^{a}	5 ± 0.4^{a}	8.3 ± 0.5^{b}	12.5 ± 1.0^{cd}	15.5 ± 1.8^{d}	22.8 ± 1.1^{e}
Seenikehel (ABB)	2.5 ± 0.5^{a}	6.3 ± 0.5^{a}	15.3 ± 1.0^{b}	27.3 ± 0.8^{c}	39 ± 1.9^{de}	44 ± 2.2^{e}

The average number of shoots ± standard error are given. Values followed by the same letter are not significantly different. Comparisons are made between the subcultures of each cultivar, using Tukey's HSD test at 5% significance.

S_o : initial culture
$S_1 - S_5$: first, second, third, fourth and fifth subcultures respectively.

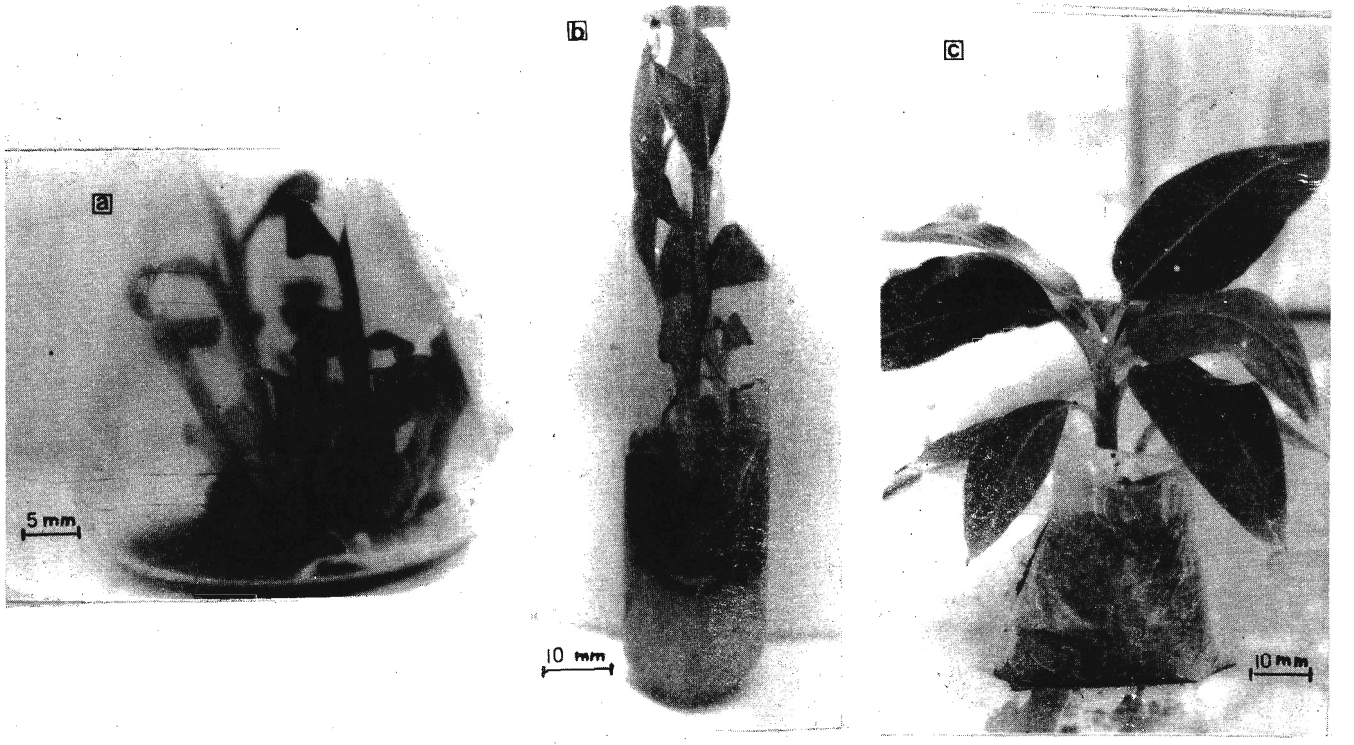

Figure 5: Regeneration of shoots into complete plants: (a) an early stage, (b) a rooted plant ready to transfer to the glasshouse, (c) a regenerated plant in the glasshouse to be transferred to the field.

Regeneration of Plants

Upon transfer of single shoots (meristems) to regeneration medium, shoots developed from all the cultivars. Within a period of 6-8 weeks, a well developed shoot was observed in all the tested cultivars. The shoots, upon transfer to rooting medium on the 6 th week, produced roots. However, in different cultivars a different time period was taken to develop a good root system. The time varied from 3-6 weeks. Complete plants were then transferred to pots and were acclimatised under glasshouse conditions (Fig. 5 a,b & c).

DISCUSSION

Shoot multiplication in ABB cultivars (Alukehel, Mondan and Seenikehel) is lower than in AAA cultivars. The highest number of shoots per culture was produced in Binkehel (AAA). From the study, it is also evident that cultivars belonging to the same group show different multiplication rates. In AAB cultivars (Embul, Puwalu, Suwandel, Kolikuttu, and Rathkehel), there is a wide variation in their rate of shoot multiplication. Thus it further confirms that the rate of shoot multiplication is cultivar dependent.[11,13]

In the use of meristem-tip culture for plant multiplication, it is important to select the correct type of sucker for successful multiplication and subsequent plant regeneration. In Sri Lanka, sword suckers are mostly recommended for banana cultivation. This was the reason for selecting sword suckers in this experiment. The use of other suckers was avoided. The importance of selecting a suitable sucker for subsequent healthy plant growth and fruit production has also been reported in other cultivars.[11,13]

Contamination and browning of the explant tissues can be considered as constraints in this technique. In fact browning of the explant tissues at the beginning is the main difficulty as contamination can be controlled with proper handling and surface sterilisation techniques. Browning is observed especially in cut surfaces of explants. However, the intensity of browning differs from one cultivar to another. But, as reported previously, it is more prominent in ABB cultivars in general. This is in agreement with the reports that *M. balbisiana* contributes more to browning.[2,4,10,14] At higher levels of browning, death of tissues could be expected as browning is due to oxidation of polyphenols.[18] This would affect the rate of shoot proliferation. This fact may be correlated to the poor rate of shoot proliferation observed in the ABB cultivars tested.

A single meristem gives rise to multiple meristems and each could be regenerated into a complete plant. It is hypothesized that newly developed meristems may arise from a group of meristematic cells or from a single cell. However in *Musa*, the former is favoured as the latter is not proven.[9,10,15] But the possibility of single cell origin cannot be ruled out. Thus, the possibility exists for generating variants or mutants from aseptically cultured shoots of *Musa* through this technique.[16,17] However, in meristem-tip culture, less variation is expected in the culture system as the meristem is more organized and cells

already have the ability to divide. This is in contrast to less organized tissues where differentiation is needed thus, a higher possibility of somaclonal variation can be expected.[19] Field studies conducted on tissue cultured plants of banana have given a rate of 13-15% somaclonal variation.[13] Experiments are in progress to study this variation in local cultivars.

The present study reveals that this technique which is already being practised successfully in several other countries, can also be used for our local cultivars of banana. This would solve the shortage of planting material for commercial cultivation of banana.

Acknowledgement

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