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Differential effect of cytokinins in the micropropagation of diploid and triploid *Musa* cultivars

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Abstract
Cytokinins showed differential effects in the micropropagation of diploid and triploid banana cultivars with respect to their genome and ploidy status. Chingan (AA) produced maximum shoot multiplication in MS medium supplemented with 8.9 µM BA while AAA cultivars (Robusta and Green red) produced maximum shoot multiplication in MS medium supplemented with 22.2 µM BA. Njalipoovan (AB) produced maximum shoot multiplication in MS medium supplemented with 8.9 µM BA and 4.7 µM KIN while AAB cultivars (Palayankodan and Nendran) yielded maximum response on MS medium supplemented with 8.9 µM BA and 4.9 µM 2iP.

Keywords: Cytokinins; *in vitro* micropropagation; multiple shoots; *Musa* cultivars.

INTRODUCTION

Bananas and plantains (*Musa* spp.) provide a staple food for many millions of people living in the humid tropics and are believed to be one of earliest plant species to be domesticated. The cultivated banana is a sterile, parthenocarpic plant selected by early farmers in south-east Asia, and thereafter maintained by vegetative propagation. Most of cultivated banana accessions are diploid or triploid and it is believed that they originated from intra- and inter-specific hybridizations between seed-bearing subspecies of *M. acuminata* (A genome donor) and *M. balbisiana* (B genome donor) (De Langhe *et al*., 2010). As a result, the various types can be classified on the basis of their genome constitution, as AA and AB (diploids), and AAA, AAB and ABB (triploids). Due to the problem of sterility and polyploidy, improvement of banana through conventional breeding has been limited. Moreover, bananas are conventionally propagated vegetatively by means of suckers. Limited availability of suckers of elite genotypes, inconvenience in handling large sized suckers, hindrance due to infection of planting materials caused by pests and diseases often becomes the major obstacles in conventional breeding.

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In vitro tissue culture propagation systems are very efficient in *Musa*. These can give high-quality, uniform plants free of disease and nematodes, and much of the planting material used in commercial plantations, and increasingly in smallholder production, comes from mass micropropagation. Shoot tip cultures have been most widely used in *Musa* (Heslop-Harrison and Schwarzacher, 2007). The rate of shoot proliferation is the most important factor of micropropagation. Apart from the influence of genotypes, shoot proliferation rate and elongation are affected by cytokinin type and their concentration. Banana cultivars of different genomic groups also behave differentially under *in vitro* conditions. However, literature available on the characterization of *in vitro* responses of Indian banana cultivars to different plant growth regulators is limited. Present study analyzed the differential effect of cytokinins in the micropropagation of diploid (AA and AB) and triploid (AAA and AAB) banana cultivars of South India.

MATERIALS AND METHODS

Young and healthy sword suckers were collected from six triploid *Musa* cultivars (2n=3x=33) namely Chingan (AA), Njalipoovan (AB), Green Red (AAA), Robusta (AAA), Palayankodan (AAB) and Nendran (AAB) from the Banana Nursery, Peringammala, Kerala, India. Suckers were washed well in running tap water and the leafy top and roots were removed. After trimming to a size of ~ 4-5 cm height, the explants were immersed in 10% (v/v) Labolene (Qualigens, India) for 15 minutes

Fig 1: a- creamy white shoot tip explant inoculated on MS medium, b- shoot tip explant turned green after two weeks, c- emergence of primary shoot within 30 days, d, e, f, g- *in vitro* shoot multiplication in hingan (AA), Robusta (AAA), Palayankodan (AAB) and Njalipoovan (AB) respectively, h- hardened shoots, i- Plantlets in field

and kept under running tap water for 40 minutes. Then, the explants were surface sterilized using 4% commercial bleach solution (final conc. 0.1% NaOCl) for 20 minutes and 0.1% (w/v) mercuric chloride for 7 minutes followed by three rinses in autoclaved double-distilled water, 5 min for each rinse. After the careful removal of primordial leaves, shoot apex of about 5-10 mm size was implanted vertically on MS medium supplemented with plant growth regulators, 30 g l^{-1} sucrose, 0.7 g l^{-1} agar and the pH of the media was adjusted to 5.8 before autoclaving. The cultures were maintained at a temperature of 25 ± 2°C with a photoperiod of 16 h/day under 50µmol m^{-2} s^{-1} light intensity provided by fluorescent lamps. The subcultures were done within an interval of 30 days and the observations were taken at 30 days interval. Then individual shoots of 5-8 cm length were separated and transferred to MS basal medium for adequate rooting and elongation of shoots. Rooted plantlets were transferred to plastic cups filled with vermiculite and hardened in the culture room conditions at 25 ± 2°C for 10 days and at 30±32°C for 2 weeks. Then the plantlets were successfully transferred to pots containing soil: sand: farmyard manure mixture (1:1:1) and maintained in the greenhouse conditions.

RESULTS

Apical shoot meristems isolated from the young and healthy sword suckers of all the cultivars showed 80-90 % survival in sterile culture conditions. After the first two weeks of inoculation, the external leaf primordia of explants turned green which were initially creamy white (Fig. 1a-b). The size of the explants increased during the initial period of culture. Microbial contamination and blackening of explants and culture medium were observed during this stage. The explants were transferred to fresh medium within a period of one week to avoid blackening at the time of culture initiation. This process of transfer has been repeated regularly for a period of one month. Primary shoots were emerged in all the survived explants within 30 days (Fig. 1c). After 30 days, subculturing was done after the removal of primary shoot. Transverse removal of primary shoots induced multiple shoots from the explants within a period of 5-6 weeks. After 6 weeks, the cluster of shoots were separated and transferred to fresh medium.

Shoot tips excised from diploid (AA) and triploid (AAA) banana cultivars showed different *in vitro* responses. Chingan (AA) showed maximum multiplication (25.29 ± 0.94 shoots/explant) on MS medium supplemented with 8.9 µM BA (Fig. 1d). But the triploid cultivars (AAA) induced maximum shoot multiplication on MS medium supplemented with 22.2 µM BA (Table 1 *[Supplementary data]*, Fig. 1e). Concentration of BA in the medium beyond 13.3 µM reduced the number of shoots in the diploid cultivar whereas; concentration of BA in the medium beyond 22.2 µM reduced the number of shoots in triploid cultivars (Fig. 2 *[Supplementary data]*). Optimum concentration for shoot length was 13.3 µM BA for both diploid (AA) and triploid (AAA) cultivars. Further

increase in the concentration of BA in the medium reduced the length of shoots.

MS medium supplemented with BA/KIN (8.9/4.7 µM) induced maximum shoot multiplication (9.43±0.48 shoots/ explant) in AB diploid (Njalipoovan) whereas MS medium supplemented with BA/2iP (8.9/4.9 µM) induced maximum shoot multiplication in AAB triploids (Fig. 3 *[Supplementary data]*). A maximum of 9.40±0.62 and 14.86±0.67 shoots/explant was produced in Palayankodan (AAB) and Nendran (AAB) respectively. In AAB cultivars, maximum shoot length was obtained from the shoots maintained on MS medium supplemented with 13.3 µM BA (Fig. 1f, 1g). However, in Njalipoovan (AB), BA / KIN (8.9/4.7 µM) induced maximum shoot length. In AB and AAB cultivars, minimum shoot length was obtained with 9.8 µM 2iP in the medium (Table 2 *[Supplementary data]*).

After three months, individual plantlets of 5-8 cm length were separated from the stock cultures and transferred to MS basal medium for proliferation of roots and further elongation of shoots. Thick, white and hairy roots were developed within 10-15 days in all the cultivars irrespective of the explants. For acclimatization, rooted plantlets were transferred to plastic cups filled with vermiculite, a commercially available sterile potting mixture (Fig. 1h). The plantlets were covered by transparent polythene sheet to maintain high humidity and within 10-15 days new leaves emerged out from all the plantlets and resumed new growth. After 2-3 weeks they were potted in a mixture of soil: sand: farmyard manure (1:1:1) and maintained in green house conditions. After 15-20 days the plants were transplanted in the open field where 90 % plants survived and developed into adult plants (Fig. 1i).

DISCUSSION

Contamination and browning of the explant tissues are the major constraints in banana micropropagation. Browning of the explant tissues at the beginning is the main difficulty as contamination can be controlled with proper handling and surface sterilization techniques. However, Titov *et al*. (2007) observed microbial contamination at the rhizomatous base of the shoot tip explants within 6-15 days after inoculation which eventually killed 85% inoculated explants. Deterioration of the cultures may also occur due to browning caused by the oxidation of phenolic compounds upon wounding of the tissues. These compounds are secreted into the medium, trapped by the agar and accumulated forming a blackened area around the explants. This may interfere with nutrient uptake, resulting in inhibition of growth and leading to deterioration of explant. In the present study, AB/AAB cultivars showed more browning when compared to AA/AAA cultivars. In the present study, explants were transferred to fresh medium within a period of one week for two months which reduced the browning of explants and culture medium.

Cytokinins are known to reduce the dominance of apical meristems and induce axillary as well as adventitious shoot formation from meristematic explants (Pandey and Jaiswal, 2002). Amongst the cytokinins, BA is the widely used, most effective and affordable cytokinin for the proliferation of multiple shoots (Johnson and Manickam, 2003). Even though, cytokinins have been known to induce shoot formation, there exist differences in the relative strength of the different types of cytokinins in shoot induction of diploid and triploid *Musa* cultivars. Banana micropropagation protocols via shoot tip culture invariably use BA (Kacar *et al*., 2010). In the present study, diploid AA cultivar produced maximum shoot multiplication in MS medium supplemented with 8.9µM BA. However, 22.2 µM BA was effective in AAA cultivars for maximum shoot multiplication. Each of the hormones tended to have an optimum concentration to achieve maximal shooting response. The better performance of 22.2 µM BA compared to 35.6 µM BA in triploid cultivars could be due to the inhibition at higher level. Similarly concentrations of BA beyond 8.9 µM BA inhibit shoot multiplication in AA cultivar. Cultivars with AB/AAB genome constitution required BA/KIN or BA/2iP combinations for optimum shoot multiplication rather than BA alone in the medium. MS medium supplemented with BA/2iP (8.9/4.9 µM) induced maximum shoot multiplication (9.40±0.62 and 14.86±0.67 shoots/explant) in AAB triploids, Palayankodan and Nendran respectively. Multiple shoot production from MS medium supplemented with BA and IAA in lower concentration was reported in two diploid cultivars of South India (Mukunthakumar and Seeni, 2005). BAP at 22.2 µM was considered optimal for shoot proliferation as well as shoot elongation from excised scalps of banana cultivars (Shirani *et al*., 2010). Cronauer and Krikorian (1985a; 1985b) obtained multiple shoot clusters from the terminal floral apices of *Musa acuminata* cv. Dwarf Cavendish (AAA), inoculated on modified MS medium supplemented with 22.2 µM BA and 10% (v/v) coconut water. Shoot apices explant also produced similar results (Cronauer and Krikorian, 1984). The high performance of BA over the other cytokinins in inducing multiplication in shoot tip cultures has been reported in different cultivars of banana (Ikram-ul-Haq and Dahot, 2007). In other plants such as *Oryza sativa, Bacopa monerria* and *Penthorum chinense* important role of BA for stimulation and proliferation of multiple shoot growth were reported (Medina *et al*., 2004; Mohapatra and Rath, 2005; Yang and Peng, 2009). The marked effects of BA on shoot formation compared to kinetin and 2iP as observed in this study may be attributed to its high stability in *in vitro* cultures which

is in agreement with Buah *et al*. (2010). BA is not easily broken down and therefore persists in the medium. It is also possible that the amount of BA get conjugated in the medium was smaller than what happened to the other plant hormones. Therefore, larger amount of BA existing in free or ionized forms in the medium are readily available to plant tissues. In the present study, number of shoots produced was higher in AA/AAA cultivars when compared to AB/AAB cultivars. Differences in the genomic constitution or phenolic contents of the cultivars might account for the variation.

The development of an *in vitro* regeneration system is an integral part and an essential pre-requisite for studies related to propagation, conservation and genetic improvement. Micropropagation is the most widely used commercialized global application of plant biotechnology in horticulture. *In vitro* propagated plants of banana and many other ornamental crops have become a commercial reality. However, micropropagation of many elite banana cultivars of local importance is still confined to research laboratories. Establishment of micropropagation protocols for local cultivars with different genome status will provide a great impetus to the global banana improvement programmes.

Abbreviations

BA: 6-benzyladenine; IAA: indole-3-acetic acid; 2iP: isopentenyl adenine; KIN: kinetin; MS: Murashige and Skoog.

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