Micropropagation of Ornamental Plant *Musa Beccarii* through Tissue Culture Technique Using Suckers and Male Buds as Explants

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Abstract: *Musa beccarii* is grouped under wild bananas and endemic in Borneo. This ornamental plant is among the most unique ornamental bananas that produce colorful and attractive flowers with small plant compared to the cultivated bananas for fruits. Micropropagation is a laboratory based tissue culture technique commonly used to propagate any plants. Micropropagation studies of these species have become important in order to increase the productivity for mass propagation in floriculture industry. This study was conducted to observe and determine the optimum sterilization procedure and media requirement for the regeneration of explants using *in vitro* method. Sterilization procedure for both suckers and male buds inflorescence for this ornamental banana were almost the same, whereas for suckers, 100% Clorox with tween 20 and 70% ethanol were used and for male buds, 70% ethanol was only used. It is found out that prolific multiple shoot formation and elongation were obtained from the suckers and male flower buds cultured on MS basal medium with 0.014mg/L BAP. Charcoal, coconut water, and gelrite (agar) were also added into the medium and PH was adjusted to 5.8. Unfortunately, the final phase was not able to perform as the plantlets were died after 5 weeks transplanting from media culture to the soil. It might be because of the sunlight that was too hot or the plant could not acclimatize to the new condition. So, the plantlets were not capable of supporting themselves in the soil.

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1. Introduction

Banana is the fourth most important fruit crop in the world. They are grown in 132 countries worldwide, more than any other fruit crop ever grown. Banana is believed to be originated from the Southeast Asia and is well known among tropical countries around the world through the trading activities. In general, banana grows well in temperature which ranges from 13ºC – 38ºC with relative humidity regime of 75-85%. The normal growth of the banana begins at 18ºC, reaches optimum at 27ºC, then declines and halts at 38ºC (Simmonds, 1966). The requirements for growing ornamental bananas are the same as growing other types of bananas. The original bananas contain rather large seeds and cannot be eaten while cultivated bananas are parthenocarpic, which makes them sterile and unable to produce viable seeds. There are many types of ornamental bananas such as *Musa ornata*, *Musa beccarii*, and *Musa velutina* (Anon, 1997). Ornamental banana such as *Musa beccarii* produces seeds but cannot be eaten. In the recent years, tissue culture propagation of banana has been utilized to increase banana production. Tissue culture is the propagation of a plant by using a plant part or single cell or a group of cells in a test tube under very controlled and hygienic conditions. Tissue culture of banana has been widely used these days due to its many advantages. Through tissue culture, elite banana varieties that are disease free can be produced because tissue cultured bananas are made under an aseptic condition where any form of contaminations from microorganisms are eliminated. Rapid multiplications of tissue cultured bananas enable early harvesting and bring a lot of benefits to the growers. The banana plants that are the products of tissue culture, have uniform size and age and the fruit bunches are of higher quality. Since tissue culture ensures continuous multiplication of plant parts in the laboratory and is not limited by the time of year or the weather, so that tissue cultured bananas are available throughout the year. This method eliminates the transmission of diseases from parent plants to offspring as the external contaminants are removed when explants are cleaned. Despite all the advantages of tissue culture, there can be a few disadvantages when it is not properly handled which can cause severe loss to a grower. For example, if a mutation occurs during mitosis, it is carried in all future divisions. A defect due to mutation could be multiplied in culture and results in thousands of cultured plants with the same defects, which can be

visible as they are planted and grown in the field. *Musa beccarii* is a type of ornamental banana under the section of *Callimusa*. The plants are small, the pseudostems are about 1-1.5m tall, sheaths are bright green, devoid of wax and somewhat polished. They are also tinged with brownish-purple at the edges. Petioles are up to 35 cm long, tightly clasping below, with erect or slightly incurved, narrowly purple and scarious margins above. Leaves are up to 100 x 30 cm, oblong-lanceolate, obtuse, the lamina halves slightly unequal, rounded or sharply cuneate at the base, bright green in color and devoid of wax. Inflorescence is small, erect, borne on a minutely hairy peduncle, and 2 cm in diameter. Basal hands are 2 - 5 in number, each bearing 1 - 3 uniseriate female flowers. Female flowers have the ovary 5 - 6 cm long, trilocular with 170 - 250 biseriate ovules. Male bud is spindle-shaped, broadest about the middle, rounded-acute at the apex strongly imbricate in the distal one third of its length. Male bract is deciduous, lanceolate, obtuse, 10 x 3 - 4 cm, almost flat, thick and leathery in the center, thinner near the edges, polished scarlet and greenish at the tip without, scarlet and duller within, persistent for a day after the flowers fall, deflexed at flowering and slightly recurved at the margins but not rolled back. Male flower is uniseriate, each 4 - 5 cm long. The stamens are 35 - 40 mm long, white, with copious chalky-white pollen. Fruit-bunch is small, erect and loosely packed, consisting of 2 - 5 hands of 1 - 3 fruits each. Fruit is shortly pedicellate, erect, cylindrical, bottlenecked at the tip, 5 - 15 x 2 cm (Simmonds, 1962). Seeds are subglobose, 4 - 5 mm in diameter, light brown in color. Somatic chromosome number is 2n = 18 (Simmonds, 1960). It takes about 6-8 months for the male buds of *Musa beccarii* to be produced. *Musa beccarii* was treated as *incertae sedis* (a term used to define a taxonomic group where its broader relationships are unknown or undefined) until Simmonds and Weatherup (1990) detected numerical taxonomic analysis of wild bananas and placed it in *Callimusa* section. However, the seed shape is not typical of *Callimusa* section and the chromosome number (2n = 18) was new to the genus *Musa*. Simmonds and Weatherup (1990) did not consider chromosome number as an important determinant of section but on this basis, Jong and Argent (2001) maintained *Musa beccarii* as *incertae sedis*. Objectives of the study were to develop *Musa beccarii* (Ornamental banana) through tissue culture population from suckers and male buds. Moreover, to make a comparison between the sterilization technique and the micropropagation process among male buds and suckers. Finally, study the micropropagation process in tissue culture of *Musa acuminata* var. *Berangan* and *Musa beccarii* (Ornamental banana).

2. Materials and Methods

2.1. Source of explants

The samples for *Musa beccarii* were prepared from Kalis Nursery and Landscaping in Janda Baik, Pahang, Malaysia. Explants that were used in this study were the suckers and male buds. For *Musa beccarii*, because of limited of sources, there were only 6 clones of suckers and 4 clones of male buds that have been cultured. Each of the samples were coded and marked.

2.2. Preparation of media

The media used in this study was the Murashige and Skoog (1962) medium, or commonly referred to as MS medium. Stock solutions of macronutrient, micronutrient, vitamin source, and Iron (Fe) sources were prepared in relatively large batches and stored in the refrigerator. Other ingredients such as Sucrose, BAP (6-Benzyl aminopurine), coconut water, and Gelrite (Agar) were also added to make the final medium.

2.3. Preparation of aseptic condition

Explants were cultured under the laminar flow cabinet. Before using the laminar flow, they were exposed to UV light for about 30 minutes to eliminate or kill all the microorganisms (pathogens). Each time the laminar flow was used, the surface of the hood wiped down with 70% alcohol. Instruments like forceps and knife (scalpel) that were going to be used were sterilized by dipping them in alcohol by flaming (burning off the alcohol) with the use of spirit lamp. Alcohol used for disinfecting instruments was 95% alcohol because it was able to burn off more easily than a more dilute form. The instruments were placed in a hot-bead sterilizer after use and then they were dipped in alcohol and flamed again before each use.

2.4. Production of *in vitro* complete plantlet from suckers

The suckers were washed to clean the remaining soil from the roots and corms. The outer layers of the suckers were removed and trimmed until the size became about 9 cm in length. Explants were put under running water for 30 minutes and rinsed with distilled water. After that, they were brought to laminar flow and were soaked in 100% Clorox and Tween-20 for 15 minutes. Then, they were rinsed with sterilized distilled water for three times, followed by soaking in 70% ethanol for 5 minutes. After this step, they were rinsed again with sterilized distilled water three times, trimmed, cut and cultured in MS media.

2.5. Production of *in vitro* complete plantlet from male buds

The outer layers of the male buds were removed up to 3-4 cm in length and put into a beaker. Explants were put under running water for 30 minutes and rinsed with distilled water for once. They were soaked in ethanol 70% for three times and rinsed with sterilized distilled water for three times. The outer layers were removed until reaching the size of 1cm × 0.5cm. Finally, explants were cut into two portions and cultured in MS media.

3. Results

In this study, only 6 suckers and 4 male buds had been cultured from the total amounts of samples from suckers of *Musa beccarii*. Only two sub-culturing in suckers and one sub-culturing in male buds of *Musa beccarii* were managed. The first batch of culture for explants (one suckers and two male buds) of *Musa beccarii* were all contaminated. In the sterilization procedure, it could be observed that the explant from suckers got contaminated easily in comparison with the male buds.

In plantlets produced from suckers, after one week of culturing, the explant turned green in color. After another week, the explant started to swell and a shoot was seen growing from the tip of the explant. The explant was then sub-cultured by excising the shoot and divided into two equal halves to induce multiple shoots formation.

In plantlets produced from male buds, the first batch of culture for male buds (two clones) of *Musa beccarii* were all contaminated because of infection by bacteria. The second batch of culture, which consisted of another two clones, however, was able to survive and this was occurred by the condition of the explants after two weeks of culture. After about two weeks, the male buds swelled a little and had turned reddish in color, just like the original color of the male buds.

Figures 1 to 7 below show the micropropagation in suckers and male buds of *Musa beccarii*. The micropropagation of the explants could be observed by counting the number of shoots formed for suckers and the number of propagules for male buds.

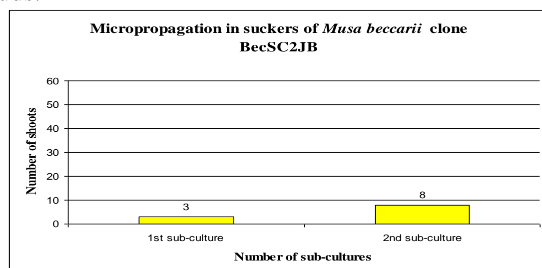

Figure 1. The number of shoots produced after two sub-cultures in suckers of *Musa beccarii* clone BecSC2JB

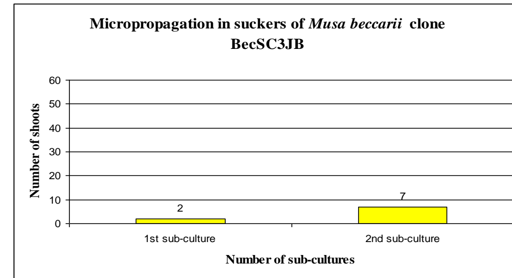

Figure 2. The number of shoots produced after two sub-cultures in suckers of *Musa beccarii* clone BecSC3JB

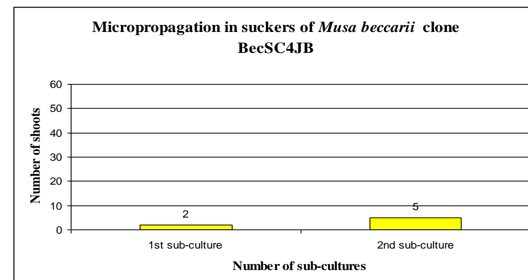

Figure 3. The number of shoots produced after two sub-cultures in suckers of *Musa beccarii* clone BecSC4JB

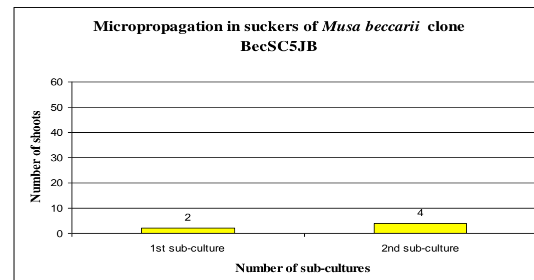

Figure 4. The number of shoots produced after two sub-cultures in suckers of *Musa beccarii* clone BecSC5JB

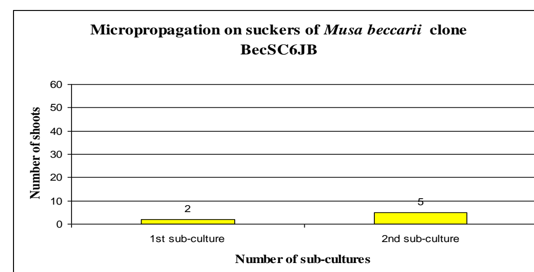

Figure 5. The number of shoots produced after two sub-cultures in suckers of *Musa beccarii* clone BecSC6JB

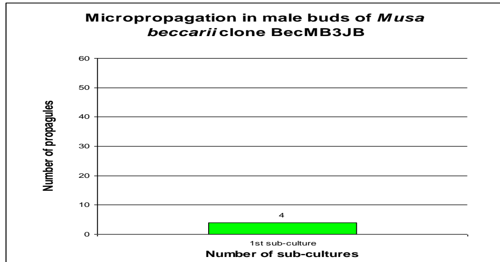

Figure 6. The number of propagules produced after one sub-culture in male buds of *Musa beccarii* clone BecMB3JB

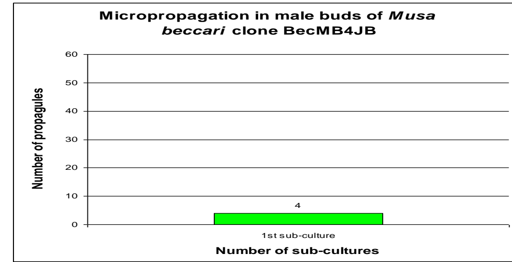

Figure 7. The number of propagules produced after one sub-culture in male buds of *Musa beccarii* clone BecMB4JB

Plate 1. *Musa beccarii* at the nursery

Plate 2. Male bud of *Musa beccarii*

Plate 3. Male bud of *Musa beccarii* before sterilization

Plate 4. Explant from male bud of *Musa beccarii* (Stage 1)

Plate 5. Explant from male buds of *Musa beccarii* after two weeks of culture (Stage 1)

Plate 6. Suckers of *Musa beccarii* before sterilization

Plate 7. Explant from suckers of *Musa beccarii*

Plate 9. Seeds of *Musa beccarii* floated when soaked in water

Plate 8. Explant from suckers of *Musa beccarii* after two weeks of culture

Plate 10. The absence of embryo in seeds of *Musa beccarii*

4. Discussions

Suckers spring up around the main plant forming a clump or "stool". The eldest sucker replaces the main plant when it fruits and dies, and this process of succession continues indefinitely. Male buds or inflorescence on the other hand, is formed from the transformed growing point in the heart of the pseudostem and undergo much of its development before emergence. Cultures initiated from explants of juvenile seedlings were easier to stabilize than those from explants of older materials. It is also important to ensure that the chosen suckers and male buds are not over matured or too old.

Size of plant materials in this study was paid much attention as it is important in determining a suitable sterilization procedure in the tissue culture of banana. The average size for suckers of *Musa beccarii* was about 80cm in length and the male bud was about 12cm in length and 4cm in wide.

It was also intended to culture embryo using the seeds of *Musa beccarii*. However, it could not be done because the seeds were not viable. They all had floated when soaked in water (Plate 9). When the seeds were opened up, no embryo was found (Plate 10). Due to time constraints, further study on embryo rescue of the seeds of *Musa beccarii* could not be done.

In this study, sterilization process was done properly as it is critical in determining the successful of a tissue culture procedure. Without proper sterilization process, explants will not be able to survive long in culture media due to presence of microorganisms that might invade them. Plant surfaces are habitats for microorganisms (Campbell, 1985). A proper sterilization process will ensure that almost all microorganisms that might present on the explants will be destroyed or killed. The sterilization steps for suckers are more extended and comprehensive compared to male buds. In sterilization of suckers, they had to undergo a series of surface sterilization using Clorox and ethanol before being rinsed in sterilized distilled water while for male buds; sterilants such as Clorox were not used. This is because suckers had originated from soils that are rich with soil-borne microbes. So, their surfaces must be thoroughly sterilized using Clorox to kill all attached microbes.

To improve wetting of the tissue surface, treatment with hypochlorite is often preceded by a detergent or alcohol wash. Ethanol partially removes hydrophobic waxes and resins which protect microorganisms from contact with aqueous sterilants. Furthermore, ethanol is a potent phytotoxic agent on its own. However, it does not kill all microorganisms; some bacteria survive when they are exposed to 96% ethanol for at least 40 min (Kunneman and Faaij-Groenen, 1988). Surface sterilization of male buds required simpler steps compared to that of suckers. This is maybe because the suckers had originated from the soil which is full of microorganisms and thus, more complex sterilization method is required.

4.1. Micropropagation

Micropropagation generally involves four distinct stages namely; initiation of cultures, shoot multiplication, rooting of *in vitro* grown shoots, and acclimatization. The first stage, culture initiation (Plates 5 and 8) depends on explants type or the physiological stage of the donor plant at the time of excision. In this stage, the innermost tissue of surface sterilized plant material was aseptically dissected and put directly into a growth medium.

In the second stage of micropropagation, the shoot multiplication is crucial and is achieved by using plant growth regulators namely; auxin and cytokinin. When the tissues started to grow in the first stage and started to form shoot, they were transferred to another medium. Difference in the manner of growth in suckers of *Musa beccarii* was observed after the initiation of culture in Stage 1. After about a week of culturing, the tissues of explants in both types of banana started to turn green in color and swollen a little. This indicated that nutrient uptake had occurred and the explant had adapted to the new environment. However, after about two weeks of culturing, the explants of *Musa beccarii,* primordial shoot started to form at the tip of the explant. The explants were then excised or cut out to initiate more shoot formation. The number of shoots increased with subsequent subcultures on the fresh culture. This was caused by the elimination of some contaminated explants. Using suckers as explants is an example of a micropropagation process via meristem culture or shoot tip culture. *In vitro* propagation through meristem culture is the best possible means of virus elimination and produces a large numbers of plants in a short span of time. After the first sub-culture, shoots started to form at the region of the apical meristem. This condition was observed in the shoot-tip culture of suckers. As the clusters of shoots are divided and re-cultured, the numbers of microshoots multiply at an exponential rate. The growing shoot multiplies and forms a dump of 3-4 shoots per clump. The pattern of multiplication and sub-culturing continues until a population of microshoots is obtained.

In the third stage, the elongated shoots derived from the multiplication stage, are subsequently rooted. After multiplication, the single shoots are separated and placed into a rooting medium. The media used in this stage is the same as MS media used in the previous stages except for the addition of activated charcoal in it. Activated charcoal was added to the medium primarily to absorb unwanted exudates, but in addition, it removed some essential chemicals from the medium such as phenolics, auxins and cytokinins. The shoots were elongated and new root came up. After about two or three months of rooting, a well-developed root system and leaves had been achieved.

The fourth stage of micropropagation involves acclimatization of *in vitro* grown plants to the natural environment. The plantlet was taken out of the culture jar and the media adhering to the root system was washed. After one week of transplanting, the plantlet had adapted to the new environment seeing to their healthy condition.

In this study, only 6 clones of suckers and 4 clones of male buds of *Musa beccarii* had been cultured. This is because, it was quite hard to find samples of ornamental banana since this type of banana is a new attractant in Malaysia besides they also needed to be bought with quite a high price. Thus, only two subcultures were done on the suckers and once in male buds of *Musa beccarii*. Plates 1, 2, 3, 4, 6, and 7 also show various details about materials in this study.

4.2. Contamination and formation of phenolic compounds

Losses can be readily ascribed to overrunning of cultures in Stage 1, which may prevent the culture of specific genotypes, or laboratory contamination due to systems failure, or infestation with mites and thrips (Blake, 1988) or poor sterile technique. Microorganisms may be rapidly expressed when they transfer to new media, for example, in the transfer from Stage 2 to 3, particularly where the concentration of salt and sucrose in the medium is reduced (Cassells, 1988; Cassells, 1991; Cassells et al., 1988).

Contamination in tissue culture can originate from two sources, through existence of microorganisms on the surface and in the tissues of explants, or through faulty procedures in the laboratory. A lot of contaminations had occurred throughout this study. Bacteria and fungi have destroyed many cultured clones of suckers and male buds.

Contamination rates are often high when explants from field-grown trees are used. From observation, I discovered that suckers were easier to get contaminated compared to male buds. The contamination rate in suckers was higher compared to that of male buds because the suckers originated from soil. Improper sterilization procedure cannot kill some of the soil microorganisms that might attach to the suckers and this may results in exogenous infection of bacteria.

According to Cassells (1991), it is more difficult to interpret the results with respect to bacterial contamination, since most often it is impossible to distinguish between endogenous and exogenous bacteria. In this study, in the case of suckers' infection by bacteria, it was impossible to determine whether the

cause of the infection was due to endogenous bacteria that had already present in the suckers even before being cultured or whether it was due to exogenous bacteria that might invade the suckers' *in vitro*.

When plant tissues are exposed to stress situations such as mechanical injury, which might happen after the isolation of an explant from the stock plant, metabolism of phenolic compounds is stimulated. This intervention leads to hypersensitivity reactions such as the release of the content of broken cells, reactions in the neighboring cells but without showing symptoms of injury themselves, and/or to the premature death of specific cells in the environment of the wound or the place of infection. It was observed that the formation of phenolic compound was less in the explants of suckers compared to that of male buds. Insufficient heating of excision tools can also results in contaminated cultures. Excision, like other actions that cause wounding, initiates the production of polyphenol oxidases which cause browning of the tissue (Vaughn and Duke, 1984; Marks and Simpson, 1990).

The production of phenolic compounds was clearly seen in the culture of male buds. The media turned brown in color and the tissues of the explants were blackened. The growth of the explant became somewhat inhibited or slowed down. This was proven by the observation made on the development of male buds in comparison with that of suckers. After a period of about nine months of culturing, a complete plantlet with an efficient root system and a distinct leaf structure had been obtained from the culture of suckers. For the male buds on the other hand, within the same period of culturing, only a flowery structure or somatic embryos were observed forming on the surface of the explants. About four to five flowery structure formed on the clump of tissues. The formation of phenolic compounds in the culture might contribute to this slow growth or development of explant from male buds.

George and Sherrington (1984) mentioned that the possibility of preventing or reducing the activity of enzymes concerned with both the biosynthesis and oxidation of phenolic, by keeping the cultures in the dark. Lowering the temperature can also reduce their activity. Most of this hypersensitivity reaction of explant was observed in the second stage of micropropagation, during the initiation of culture.

5. Conclusions

There are many advantages of micropropagation namely; the production of more uniform plants, produced plants often grow faster and show improved vigor, and plants tend to mature earlier than when propagated by seed. Banana cultivation and production are threatened by many pests and diseases. By using tissue culture, this problem can be reduced since production of disease-free plantlets can be achieved.

Ornamental banana is a new attractant in Malaysia. Planting material of ornamental plants is in great demand for commercial production as well as for domestic gardens and landscaping. So, if the planting material for ornamental banana can be produced in high amount through micropropagation, a lot of profits can be obtained.

Ornamental plants are produced mainly for their aesthetic value, thus the propagation and improvement of quality attributes such as leaf types, flower color, longevity and form, plant shape and architecture, and the creation of novel variation are important in terms of economic goals for floriculturists. Successful *in vitro* propagation of ornamental plants is now being used for commercialization.

Tissue culture population of *Musa beccarii* can be developed using suckers and male buds as explants. Multiple shoots of *Musa beccarii* were obtained. The development or growth of explant from suckers was faster compared to male buds. Within the same period of culturing, a complete plantlet can be obtained from the culture of suckers but only somatic embryos were obtained in the culture of male buds. Different sterilization technique needs to be used in culturing male buds and suckers since both explants have different degree of contamination rates because of the difference in size and background. Different types of micropropagation process can be observed in male buds and suckers. The male buds undergo direct somatic embryogenesis while the suckers were derived from the organogenesis pathway. Only two sub-cultures were done on the suckers and once on male buds of *Musa beccarii*. From observation, it can be concluded that suckers were more prone to contamination than male buds. The formation of phenolic compound on the other hand, was more visible in male buds.

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