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Effect of Phenological Growth Stage on Establishment of *In-vitro* Cultures of Bael (*Aegle marmelos* (L.) Corr.)

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ABSTRACT: *Bael (Aegle marmelos (L.) Corr.) is a medicinal fruit tree species belongs to the family Rutaceae grown in South Asian countries including India, Sri Lanka and Bangladesh. It is an underutilized fruit species in Sri Lanka, although it has food as well as medicinal value with a good economic potential. Popularizing bael as a profitable cash crop is often hindered by the limited availability of high quality planting material. In Sri Lanka, five elite bael accessions namely Beheth Beli, Paragammana, Mawanella, Rambukkana and Polonnaruwa Supun have been identified and used for mass propagation through budding and grafting. But this effort is often hampered by many limitations faced in large scale production. Micropropagation is an alternative technique to produce clonal plants in large scale. However, the complex phenological behaviour of the bael trees could affect its success. Therefore, the present study was undertaken to identify the correct phenological stage of bael trees to collect explants for the micropropagation. Leaves and twig explants were collected for micropropagation from five elite bael accessions during the period of July, 2016 to June, 2017 on monthly basis to capture the best phenological stage. The Beheth Beli tree and grafted plants of other four accessions were established at the Fruit Crop Research and Development Institute, Department of Agriculture, Sri Lanka. The surface sterilized explants were established on Murashige and Skoog medium supplemented with 1 mg/L of 6-Benzylaminopurine, 3% sucrose, solidified with phytogel. A successful organogenesis was only observed in explants collected in the months of April, May and June (39 to 68 %) where there was no significant difference in success was observed between the leaf and twig explants ($P>0.05$). However, grafted bael accessions exhibited a significantly different mean success percentage in organogenesis where Paragammana and Rambukkana accessions showed a high success for leaf explants and Mawanella and Rambukkana accessions showed a high success for twig explants. These results could be readily employed to multiply the elite bael accessions in Sri Lanka.*

Keywords: *Bael, clonal propagation, explants, phenology, rapid multiplication, tissue culture*

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INTRODUCTION

Aegle marmelos (L.) Corr. of family Rutaceae, commonly known as bael, is a medicinal fruit tree fruit species (Chanda *et al*., 2008). Bael is native to India and also found in South Asian countries such as Sri Lanka and Bangladesh, South-East Asia and Egypt (Singhal *et al*., 2011). Ripened fruit is the economically important plant part of bael. Whereas, all the plant parts have important medicinal properties (Seth, 2003; Benni *et al*., 2011). The pulp of the ripened fruits is delicious and can also be processed into value added products such as jam, syrup, sweets and herbal drinks (Baliga *et al*., 2011; Morton, 1987). Bael is considered as a sacred tree in India and often grown as a sacred tree near the temples dedicated to 'God *Shiva*' (Singhal *et al*., 2011). The fruits and other plant parts of bael contain important phytochemicals such as tannin, courmarin, aegelinol and marmelocin and they exhibit various medicinal properties ranging from laxative, anti-proliferative, anti-diabetic and anti-cancerous capabilities (Suvimol and Pranee, 2009; Lambole *et al*., 2010).

Bael is a well-established medicinal fruit tree species and the selections from the wild germplasm have led to larger fruit sizes and superior fruit qualities. Elite bael plants have been located and attempts have been made to multiply them for large scale cultivations in India (Pati *et al*., 2008). However, propagation using seeds is often hampered by the extreme genetic heterozygosity due to open pollination, lower seed germination rates and difficulty in collecting and processing seeds from mature fruits. The budding and grafting are also problematic as the associated operations are skilled-labor intensive and tedious, and due to seasonality of the vegetative parts.

Micropropagation techniques have been successfully employed for rapid multiplication of the superior bael plants (Pati *et al*., 2008). The cotyledons (Hossain et *al*., 1994; Nayak *et al*., 2007; Pradeepa Devi *et al*., 2014; Prematilake *et al*., 2006), nodes (Pati *et al*., 2008) and the axillary buds (Ajithkumar and Seeni, 1998) have also been successfully used as explants in bael micropropagation. However, phenological growth stage of the tree subjected to the collection of explants has a greater influence on the percentage success in micropropagation (Raghu *et al*., 2007). Bael has marked phenological growth difference with respect to the rainfall, temperature and fruiting (Kishore *et al*., 2017). Rhagu *et al*. (2007) reported that October to December is the best time period to collect explants as the plant is at its best phenological stage for higher success rate of micropropagation in Kerala, India.

Although bael is a popular medicinal fruit tree species in Sri Lanka, its full economic potentials have not been exploited yet and considered as an underutilized fruit species (Pushpakumara *et al*., 2007). Lack of research (Arseculeratne *et al*., 1985) and limited availability of high quality planting material often hinder the establishment of bael as a profitable cash crop in Sri Lanka. The Fruit Crop Research and Development Station (FCRDS) of the Department of Agriculture, Sri Lanka has identified superior bael trees to be used as mother plants for large scale cultivation. However, due to the limitations in the production of planting materials through budding and grafting, the FCRDS is not in a position to provide the true-to-type bael plants for the countrywide growers. Therefore, the present study was conducted to identify the best phenological growth stage of the elite bael accessions to harvest the explants for mass propagation through micropropagation with the objective of adequate and timely provision of clonal plants to the Sri Lankan growers.

MATERIALS AND METHODS

Five elite Sri Lankan bael accessions namely *Beheth Beli* (BB), *Paragammana* (PA), *Mawanella* (MA), *Rambukkana* (RA) and *Polonnaruwa Supun* (PS), which have been selected for large scale production of planting materials, were used to collect the explants for developing a protocol for micropropagation.

The study was conducted at the Tissue Culture Laboratory of the Department of Crop Science, Faculty of Agriculture, University of Peradeniya, Sri Lanka. Leaves and twigs were used as the explants. Explants from the mother plant of BB were collected from Fruit Research and Development Station (FCRDS) of the Department of Agriculture, Gannoruwa, Sri Lanka (GPS coordinates: 7.277006, 80.595299) because of close proximity to the study location. As the original mother plants of the other four accessions are located at distant locations from the study site, the explants were collected from the grafted plants established at FCRDS. A total of 50 immature leaves and 50 tender twigs were collected from each plant and immediately transferred to the laboratory for micropropagation. The samples were collected once in a month from July, 2016 to June, 2017 to represent the phenological growth stages of the bael trees within a year. The phenological stages of the plants were recorded during the collection of the plant materials for tissue culture experiments.

Preparation of explants, sterilization and micropropagation

The dried/dead and infected parts were removed from the intended explants. The twigs and leaves were isolated and the bael twigs containing at least three to four internodes were used for culturing. The leaves and internodal explants were thoroughly cleaned using commercially available detergent mixture and smooth bristles and rinsed using running tap water for ten minutes. After washing few times the plant parts were soaked in distilled water approximately for one hour. Then subsequent steps were followed for further sterilization to avoid the bacterial and fungal infections.

The plant parts were soaked in a fungicide solution (2.5%) for 2 hours and washed carefully with distilled water. Then they were transferred into a wash bottle which consisted of 10% Sodium Hypochlorite (NaOCl) and a drop (1 mL) of Polyethylene Glycol Sorbitan Monolaurate (Tween 20/T20) and shaken for 10 min. Then bleach solution was drained off and washed three times carefully with autoclaved water. That step was repeated with only 10% NaOCl and cleaned plant parts were transferred to a vessel containing distilled water.

The MS (Murashige and Skoog, 1962) medium supplemented with 1 mg/L of BAP was used for initiating the cultures. Leaf pieces at the size 1 cm^2 (approximately) containing the mid ribs were isolated and placed on the medium. A gentle press was applied to have an even contact between the abaxial (lower) surface of the leaf and the medium. The twigs were inserted into the medium in upright position. The twig cultures were incubated at 16 hours of light (flux density) at 25±2 °C of temperature with 75% of relative humidity. The leaf cultures were incubated inside a dark cabinet at 26±2 °C until initiating the shoots.

Data collection and analysis

The contaminated and uncontaminated cultures were counted. The number of successful leaf and intermodal explants of elite bael accessions were counted and subjected to ANOVA Procedure in the Statistical Package SAS 9.1 (SAS Institute, Cary, NC, USA).The phenological stage of the plant during explants collection was also recorded.

RESULTS

The phenological growth stages of the bael tree BB for twelve months are given in Table 1. The time period between July to March could be considered as reproductive phase, however, sparse shoot generation was seen in November because of the rainfall. The vegetative phase started with in March with the inter-monsoonal rains, and lasted till June. The reproductive phase was started in June with the onset of floral bud break and the initiation of inflorescences. The successful organogenesis, measured as the % explants that exhibited shoot development, was not observed for any of the leaf or twig explants collected from BB in the months July, 2016 to March, 2017 (*i.e.* reproductive phase; Plate 1; Table 1). The explants harvested from BB during April to June, 2017 (*i.e.* vegetative phase) produced successful organogenesis in the range of 62-68% (Table 1).

The percentage organogenesis of the four grafted elite bael accessions revealed that the successful organogenesis has occurred only in the explants harvested from March to June, 2017. The accessions PA and RA yielded significantly higher percentage ($P<0.05$) of organogenesis (55.0% and 57.7%, respectively) than the other two for leaf explants. The lowest mean success percentage ($P<0.05$) for leaf explants was observed for PA (44.3%), however, MA and RA exhibited a higher success percentage for twig explants (53.3% each; $P<0.05$) and the lowest was observed for PA and PS (49.7% and 49.0%, respectively; $P<0.05$); Table 2). If the percentage success of the leaf and twig explants were compared for the months April, May and June, 2017, no significant differences ($P>0.05$) were observed in the % success of the leaf and twig explants obtained during the months of April, May and June in 2017 (Table 3).

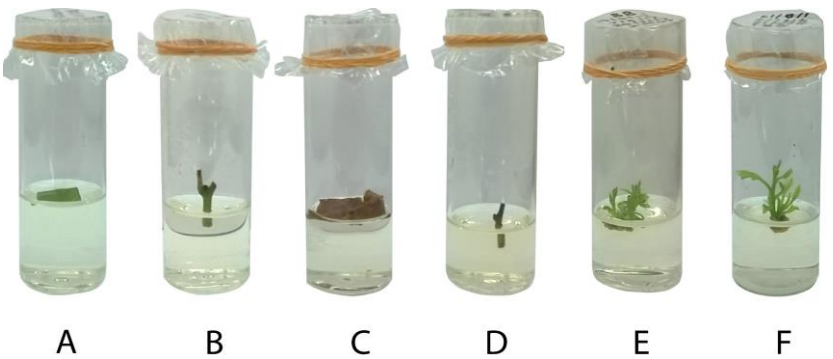

Plate 1. The representative images of the explants at the initial establishment and after eight weeks. A and B: Leaf and twig, respectively, at the initial establishment, C and D: Leaf and twig (collected before April), respectively, with no development, E and F: Leaf and twig (collected after April), respectively, with development after eight weeks.

Table 1. Phenological growth stage and the percentage micropropagation success of the explants collected from the bael accession *Beheth Beli* in 12 months

Month, Year	Phenological Growth Stage[a]	% Success	
		Leaves	Twigs
Jul-16	Leaf development is completed, leaves are matured and full bloom	-[b]	-
Aug-16	Small fruits can be seen, hardened branches	-	-
Sept-16	Fruit development and enlargement	-	-
Oct-16	Fruit development and enlargement	-	-
Nov-16	Fruits development, new shoots are rarely emerged with the inter monsoonal rainfall	-	-
Dec-16	Fruit development	-	-
Jan-17	Fruits reached the maximum size	-	-
Feb-17	Senescence of mature fruits is started	-	-
Mar-17	Senescence of mature fruits and vegetative buds breakup with inter monsoonal rainfall, vegetative bud development	-	-
Apr-17	More vegetative buds breakup with inter-monsoonal rain fall, vegetative bud development	65%	62%
May-17	Vegetative bud development with monsoonal rain fall	68%	65%
Jun-17	Vegetative development with monsoonal rain fall, floral bud initiation and breaking, inflorescence development	67%	68%

[a]*FCRDS records and, observations made in the present study.*
[b]*Did not undergo organogenesis and subsequently got contaminated.*

Table 2. Percentage success in micropropagation in the grafted bael accessions for April, May and June of 2017*

Accession	% Success of explants	
	Leaves	Twigs
PA	55.0[a]	49.7[b]
MA	51.3[b]	53.3[a]
RA	57.7[a]	53.3[a]
PS	44.3[c]	49.0[b]

Within a column, means followed by the same superscript are not significantly different at P=0.05; No explants showed organogenesis or shoot proliferation from July 2016 to March 2017; PA – Paragammana; MA – Mawanella; RA – Rambukkana; PS - Polonnaruwa Supun

Table 3. Percentage micropropagation success for all bael accessions combined in monthly basis

Month, Year	% Success of explants	
	Leaves	Twigs
Other months 2017	0.0[b]	0.0[b]
April, 2017	55.8[a]	55.2[a]
May, 2017	53.3[a]	51.8[a]
June, 2017	47.2[a]	47.0[a]

Within a column, means followed by the same superscript are not significantly different at P=0.05.

DISCUSSION

The phenological stage of the plants is important for deciding the effective fruiting season and to collect the vegetative parts as planting material (Salinero *et al*., 2009). During flowering and fruiting of the plants, the internal hormonal profiles often hinder the effective regeneration capabilities. However, if the plant parts are taken from actively growing trees, regeneration can be achieved swiftly with higher success rates (Raghu *et al*., 2007). The phenology of bael is complex and vegetative and reproductive phases are overlapped (Kishore *et al*., 2017). In India, the bael phenology has been correlated with success rates in micropropagation (Raghu *et al*., 2007). However, such studies have not been conducted in Sri Lanka to the best of our knowledge. Hence, the results of the present study possess paramount significance in deciding the exact phenological stage of bael to be used for a successful micropropagation.

The months April, May and June, which are immediately after the fruiting season, could be considered as the best season to collect the explants of bael (Tables 1 and 3). Although limited % of success have been observed for other phenology-wise vegetatively inactive stages in India, present study did not show any organogenesis from the explants collected from such months. This could be due to the climatic pattern experienced by the bael mother plants established at Gannoruwa, Sri Lanka. As a future direction of the study it would be better to study the phenological impact on the micropropagation success of the explants collected from diverse locations in Sri Lanka with variable climates.

According to the present analysis, all the bael accessions yielded explants during April to June for successful micropropagation implying that all of them have common phenological behavior (Table 3). Although, sparse new shoots emerged due to the availability of rainfall, they are not useful as a source of explants for micropropagation (Table 1 and 2) during other months. Some of the grafted accessions showed marked significant variation in the success of organogenesis for leaf or twig explants indicating that they have variable responses due to genetic differences (Table 2). It is also evident from the present study that leaf and twig explants can be successfully employed in micropropagation when they are at the correct physiological stage.

CONCLUSIONS

The micropropagation using leaf and twig explants of five elite bael accessions in Sri Lanka collected from monthly phenological stages revealed that the successful micropropagation could be done if the explants are harvested during April to May, immediately after the fruiting season of the plant. Both leaf and twig explants exhibited similar success rates in undergoing direct organogenesis and axillary bud proliferation. These results could be successfully employed in mass production of clonal bael plants from elite accessions to establish the species as a profitable cash crop in Sri Lanka.

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