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In vitro propagation of Aegle marmelos (L.) Corrêa via nodal cultures: effects of auxin– cytokinin combinations and acclimatization substrates

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Short Report

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Abstract

Aegle marmelos (L.) is a medium-sized deciduous tree. It is a subtropical plant that can adapt to a wide range of habitats and can be grown worldwide, including in India, Thailand, Laos, Vietnam. We optimized an *in vitro* micropropagation protocol using nodal explants. On MS medium, the combination 1.5 mg L^{-1} 6-benzylaminopurine (BAP) + 0.5 mg L^{-1} indole-3-butyric acid (IBA) produced the highest number of shoots (4.90 per explant at 8 weeks), significantly higher than the hormone-free control. For rooting, 1.0 mg L^{-1} α-naphthaleneacetic acid (α-NAA) + 1.5 mg L^{-1} IBA yielded the greatest root number (3.23) and length (1.49 cm) at 8 weeks. During acclimatization, plantlets transferred to rice husk:coconut fiber:loam (1:2:2) achieved 100% survival and vigorous growth. This reproducible protocol supports large-scale multiplication of disease-free *A. marmelos* plantlets for cultivation and conservation.

Introduction

Aegle marmelos (L.) (*A. marmelos*) Corrêa (Rutaceae), commonly known as Bael (Bilva or Bhel), also called quince in Bengal, pomme d'or,. Bael is medium-sized deciduous tree. It is a subtropical plant that can adapt to a wide range of habitats and can be grown worldwide, including in India, Thailand, Laos, Vietnam. (Khanal et al., 2023; Pathirana et al., 2020). There are many organic compounds in Bael, such as alkaloids, terpenoids, coumarins, phenylpropanoids, tannins, polysaccharides, and flavonoids (Biswas et al., 2023; Savita et al., 2021). Consequently, all parts, including roots, bark, leaves and fruit are used medicinally to support the treatment of diseases such as diabetes, eye infections, constipation, respiratory disorders, arthritis (Khanal et al., 2023). Additionally, Bael is utilized as a food source for making drinks, candies, jams, tea, and more. Beyond its food and medicinal value, Bael is recognized for its relatively high photosynthetic efficiency and oxygen release capacity compared to other trees (Khanal et al., 2023; Pathirana et al., 2020).

Due to its dual role as a medicinal herb and raw material for food processing, the demand for raw materials from this plant is increasing day by day. To meet the growing demand for Bael, it is essential to employ new propagation methods, such as *in vitro* culture technology, to establish a sustainable seed source. Micropropagation, or *in vitro* propagation, offers a promising approach for the large-scale multiplication of elite and disease-free genotypes of medicinally important plants. Tissue culture techniques provide year-round propagation, genetic uniformity, and the potential for conservation of endangered germplasm (Hanchana et al., 2017). Several reports on *A. marmelos* have demonstrated successful shoot induction and proliferation using cytokinins such as 6-benzylaminopurine (BAP), often in combination with auxins like indole-3-butyric acid (IBA) or indole-3-acetic acid (IAA). For instance, nodal explants cultured on MS medium supplemented with 0.5 mg L^{-1} BAP achieved multiple shoot formation, whereas rooting was promoted by IBA or NAA supplementation (Bahnasy et al., 2025). Among the growth stimulants, a BAP concentration of 2 mg/L yielded the best results for shoot formation. The combination of IAA with BAP was found to increase multi-shooting. For callus formation, it is often combined with various auxin and cytokinin growth stimulants (Mandal et al., 2021). However, there are few studies on the combination of growth hormones in the *in vitro* propagation of Bael.

Moreover, acclimatization—an essential step for transitioning *in vitro* plantlets to soil—has received comparatively little attention, and systematic evaluations of substrate compositions are lacking.

Given these gaps, the present study was undertaken to (i) optimize shoot multiplication and rooting responses of nodal explants of *A. marmelos* under different auxin–cytokinin regimes, and (ii) evaluate the survival and growth performance of plantlets during acclimatization on different substrate mixtures. The outcomes are expected to provide a reproducible and efficient protocol for the micropropagation and conservation of this important medicinal species.

Materials and Methods

Sample Description

The plant material used in this study was *Aegle marmelos* (L.) Corrêa, a member of the Rutaceae family. Taxonomic identification of the species was carried out by Ass. Pro. Dr Sy Danh Thuong, Department of Botany, Thai Nguyen University of Education, Vietnam. A voucher specimen (No. AM-TNUE-2022) has been deposited in the Herbarium of Thai Nguyen University of Education, Vietnam, and is available for future reference.

Plant material was obtained with permission from the Faculty of Biology, Thai Nguyen University of Education. The collection did not involve endangered or protected populations, and thus no special permits were required. Sampling was conducted with authorization from the governing body. All procedures complied with institutional, national, and international guidelines for research involving plants.

The used research materials were *in vitro A. marmelos* shoots in our previous research (Hiep et al., 2022). Stem nodal explants (3–4 cm long, each bearing one or two axillary buds) were excised from healthy, *in vitro*–raised seedlings of *Aegle marmelos* (L.) Corr. after 3 weeks of culture. The explant source was maintained under sterile conditions in the laboratory at Faculty of Biology, Thai Nguyen University of Education, TNU, and voucher/reference plantlets were retained *in vitro* for future verification.

Surface Sterilization

Explants were sterilized by immersion in 70% (v/v) ethanol for 30 seconds, followed by 10-minute treatment in 0.1% (w/v) mercuric chloride ($HgCl_2$) containing a few drops of Tween-20. After thorough rinsing (3 × 5 minutes) in sterile distilled water, explants were trimmed under aseptic conditions and transferred to culture media.

Culture Media and Hormone Treatments

Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) supplemented with 30 g L^{-1} sucrose and solidified with 7 g L^{-1} agar (pH adjusted to 5.8 before autoclaving at 121°C for 20 minutes) was used as the basal medium unless otherwise noted. The control medium was hormone-free MS medium.

Culture conditions

Experiments were carried out under the conditions of temperature 22–25^{o}C, light intensity of 2,000 lux, lighting time 14 hours, humidity of the culture room 50–60%.

Shoot Induction and Multiplication

After removing the leaves, the shoots were divided into one-node pieces. During the multiplication stage, node explants were cultured on a hormone-free MS medium or supplemented with hormone BAP and IBA (BAP at 0.5, 1.0, 1.5 and 2.0 mg/L and IBA 0.5 mg/L) or IAA and BAP (IAA at 0.5, 1.0, 1.5 and 2.0 mg/L and BAP 1.0 mg/L) for multiplication. Data were collected after six, eight weeks of culture.

Rooting of In vitro Shoots

For root induction, shoots 1.5- 2.0 cm in length that are transferred to MS medium contain IBA 1.0 mg/L and α-NAA (0.3, 0.5, 0.7 and 0.9 mg/L) to make roots. Data were collected after 6, eight weeks of culture. Cultures were maintained under identical environmental conditions as shoot induction. Rooting responses were recorded after six eight weeks: percentage rooted, number of roots per shoot, and average root length.

Acclimatization (Hardening)

Healthy plants with well-developed roots were washed, and placed in trays filled with sterilized coir for survivability survey. During this period, Bael plants reaching the desired criteria of 3–5 leaves, 4 cm height, and 6 cm long stalks will be selected for further experimentation. Following the survey, these chosen plants will be transferred from coir to three different potting soil mixtures: the formula 1 includes: rice husk + coconut fiber + loam (1:1:1 ratio); the formula 2 include: rice husk + coconut fiber + loam (1:2:2 ratio); the formula 3 including: rice husk + coconut fiber + loam (1:2:3 ratio). Plant performance will be monitored over a 40-day follow-up period, focusing on key criteria: survival rate, number of leaves, plant height, plant color, assessment of growth ability, overall development. The follow-up period is 40 days.

Experimental Design and Statistical Analyses

Design: Completely randomized design with three replicates per treatment; each replicate consisted of 10 explants.

Data recorded: For shoot multiplication—percentage of responding explants, mean number of shoots per explant, shoot length; for rooting—percentage rooting, mean number of roots per shoot, root length; for acclimatization—survival rate at 4 and 6 weeks.

Analysis: Data were analyzed via one-way ANOVA using SPSS v26. Post-hoc comparisons were performed using Duncan's Multiple Range Test (DMRT) at $\alpha = 0.05$. All values are presented as mean ± standard deviation (SD).

Data presentation: Within each Tab. or Fig., means followed by different letters indicate significant differences at $p < 0.05$.

Results and Discussion

Effect of plant growth regulators on shoot multiplication

Effect of both IBA and BAP to produce multiple shoots

Using 30 samples for each experiment and repeated 3 times, the result of effect of IBA and BAP to produce multiple shoots is shown in Table 1 and Fig. 1.

Table 1
Effect of IBA and BAP to produce multiple shoots

BAP conc. (mg/L)	IBA conc. (mg/L)	Shoots per explant	Shoots height (cm)	Shoots quality
After six weeks of culture				
0	0	$1.23^{a} \pm 0.15$	$2.51^{b} \pm 0.20$	+
0.5	0.5	$1.82^{b} \pm 0.13$	$1.31^{a} \pm 0.43$	+
1.0	0.5	$3.46^{c} \pm 0.44$	$1.22^{a} \pm 0.11$	+++
1.5	**0.5**	**$4.13^{d} \pm 0.06$**	**$1.37^{a} \pm 0.07$**	**+++**
2.0	0.5	$1.55^{ab} \pm 0.31$	$1.62^{a} \pm 0.54$	+
After eight weeks of culture				
0	0	$1.33^{a} \pm 0.25$	$2.65^{b} \pm 0.39$	++
0.5	0.5	$2.24^{b} \pm 0.25$	$1.35^{a} \pm 0.30$	++
1.0	0.5	$4.01^{c} \pm 0.58$	$1.38^{a} \pm 0.30$	+++
1.5	**0.5**	**$4.90^{d} \pm 0.10$**	**$1.42^{a} \pm 0.08$**	**+++**
2.0	0.5	$2.30^{b} \pm 0.43$	$1.38^{a} \pm 0.28$	+

Note: Mean ± SD followed by the different letters within each column are significantly different according to Duncan's multiple range test at $P < 0.05$

The results in Table 1 and Fig. 1 demonstrate that the combination of IBA and BAP has a significant effect on the ability to produce multiple shoots in Bael. After six weeks, the negative control (MS0 media without plant growth hormones) exhibited the lowest number and height of shoots, shoots quality were weak (small shoots with light green leaves). Conversely, the other treatments showed a higher number and greater height of shoots than the negative control. By the 8-week mark, the most effective formulation for generating multiple shoots was determined to be MS media supplemented with 0.5 mg/L IBA and 1.5 mg/L BAP, resulting in 4.90 shoots with an average height of 1.42 cm. The shoots exhibited robust growth with vibrant green leaves. In contrast, the alternative formulation had a higher number of shoots per sample, and the height of the shoots surpassed that of the negative control, shoots quality is the best (Fat shoots with big green leaves). Gandhi et al. (Gandhi et al., 2018) used the medium fortified with 11.11 µM/L BAP and 1.07 µM/L NAA for the initiation and multiplication of shoots with highest mean number of shoots (24.20 ± 3.85) and maximum mean length of the shoots (6.02 ± 0.64). In 2008, Pati et al. used MS medium supplemented with 8.84 µM BAP and 5.7 µM IAA to treat multiple shoots. The maximum number of proliferated shoots was 9.0 shoots/explant (Pati et al., 2008).

Effect of both IAA and BAP to produce multiple shoots

The effects of IAA and BAP on the production of multiple shoots are presented in Table 2 and Fig. 1.

Table 2
Effect of IAA and BAP concentrations on shoot multiplication

BAP conc. (mg/L)	IAA conc. (mg/L)	Shoots per explant	Shoots height (cm)	Shoots quality
After six weeks of culture				
0	0	$1.20^{a} \pm 0.22$	$2.52^{b} \pm 0.26$	+
1.0	0.5	$1.95^{a} \pm 0.09$	$1.13^{b} \pm 0.05$	+
1.0	1.0	$3.20^{b} \pm 0.59$	$1.21^{a} \pm 0.08$	+++
1.0	**1.5**	**$4.00^{b} \pm 0.29$**	**$1.65^{a} \pm 0.28$**	**+++**
1.0	2.0	$1.55^{a} \pm 0.31$	$1.28^{a} \pm 0.07$	+
After eight weeks of culture				
0	0	$1.28^{a} \pm 0.21$	$2.86^{b} \pm 0.25$	++
1.0	0.5	$2.12^{a} \pm 0.09$	$1.10^{b} \pm 0.04$	++
1.0	1.0	$3.56^{b} \pm 0.39$	$1.35^{a} \pm 0.05$	+++
1.0	**1.5**	**$4.20^{b} \pm 0.74$**	**$1.70^{a} \pm 0.10$**	**+++**
1.0	2.0	$1.88^{a} \pm 0.37$	$1.35^{a} \pm 0.07$	+

Note: Mean ± SD followed by the different letters within each column are significantly different according to Duncan's multiple range test at $P < 0.05$

At the end of eight weeks, the formulation that demonstrated the highest potential for producing multiple shoots was the basic MS medium enriched with 1.0 mg/L of BAP and 1.5 mg/L of IAA, resulting in an average of 4.20 shoots per sample, with a shoot height measuring 1.70 cm. The formulation with the fewest multiple buds was the MS medium supplemented with 1.0 mg/L of BAP + 2.0 mg/L of IAA (yielding a shoot count of 1.88 per sample and a shoot height of 1.35 cm). All experimental treatments exhibited higher shoot counts per sample and greater shoot heights than the control (which had 1.28 shoots per sample and a height of 2.86 cm).

According to our research (Hiep et al., 2022), Bael grew well and produced multiple shoots in the control medium supplemented with both 0.5 mg/L IBA and 1.0 mg/L Kinetin. Compared our previous research, this experiment gave better results in terms of both the number and length of shoots. Thus, it can be

seen that Bael grows and creates multiple shoots well on medium supplemented with growth hormones, especially on control medium with both 1,0 mg/L BAP and 0,5 mg/L IBA shoots reached 4.13 cm, shoot height is 1.37 cm. All shoots were fat and had big dark green leaves.

Effect of both α-NAA and IBA on rooting

The effectiveness of α-NAA and IBA in promoting root development in Bael is visually represented in Table 3 and Fig. 2.

Table 3
Effect of α-NAA and IBA concentrations on roots multiplication

α-NAA Conc. (mg/L)	IBA Conc. (mg/L)	Roots/sample	Root length (cm)	Roots quality
After six weeks of culture				
0.0	0.0	$0.55^{a} \pm 0.25$	$0.65^{b} \pm 0.44$	+
1.0	0.5	$0.85^{b} \pm 0.51$	$0.38^{a} \pm 0.31$	+
1.0	1.0	$1.00^{c} \pm 0.36$	$0.87^{c} \pm 0.65$	++
1.0	**1.5**	**$1.52^{d} \pm 0.20$**	**$0.82^{c} \pm 0.59$**	**+++**
1.0	2.0	$0.61^{a} \pm 0.50$	$0.33^{a} \pm 0.31$	+
After eight weeks of culture				
0.0	0.0	$1.04^{a} \pm 0.31$	$0.73^{a} \pm 0.26$	+
1.0	0.5	$1.30^{b} \pm 0.35$	$0.85^{b} \pm 0.44$	+
1.0	1.0	$1.84^{c} \pm 0.51$	$0.94^{c} \pm 0.40$	++
1.0	**1.5**	**$3.24^{d} \pm 0.51$**	**$1.49^{d} \pm 0.23$**	**+++**
1.0	2.0	$1.25^{b} \pm 0.51$	$0.67^{a} \pm 0.40$	+

Note: Mean ± SD followed by the different letters within each column are significantly different according to Duncan's multiple range test at $P < 0.05$

Though roots emerged in all media, their enthusiasm varied greatly depending on the environment. As illustrated in the Fig. 2, even subtle changes can significantly impact Bael's root development. The synergistic power of α-NAA and IBA shone through in this experiment, revolutionizing Bael plant root development. After eight weeks culture, a mere 1.0 mg/L α-NAA and 1.5 mg/L IBA in the culture medium produced dramatically improved root growth compared to previous studies, highlighting the effectiveness of this precise hormone combination. All other tested formulas (1.0 mg/L α-NAA and 0.5 mg/L IBA, 1.0 mg/L α-NAA and 1.0 mg/L IBA, and 1.0 mg/L α-NAA and 2.0 mg/L IBA) significantly

outperformed the negative control. None of the formulas containing growth hormones displayed root growth like the 1.0 mg/L α-NAA and 1.5 mg/L IBA combination which produced the most robust and extensive root system observed in this experiment.

According to our research (Hiep et al., 2022) In the MS0 medium supplemented with IAA 1.0 mg/L, the plants gave strong growth with the number of roots/sample reaching 0.60 roots/acre and the root length reaching 0.90 cm. Pati et al. also used MS medium supplemented with 5.7μM/l IAA to induce rooting, resulting in 100% of shoots rooting (Pati et al., 2008). In the study of Hossain et al., the author used MS supplemented with 0.5 mg/L IBA for rooting, *in vitro* rooting seedlings were transferred to the natural soil environment (Hossain et al., 1997). Hanchana et al. used MS basal medium together with 1.0 mg/L IBA combined with 1.5mg/L IAA for rooting of Bael, resulting in 62% rooting shoots (Hanchana et al., 2017). Thus, compared to previous studies, concentrations of 1.0 mg/L α-NAA and 1.5 mg/L IBA gave the best rooting results with 3.23 roots/sample and root length of 1.39 cm after eight weeks of culture.

The results of the investigation of suitable substrates to bring Bael in vitro to grow in a greenhouse

Before carrying out planting on suitable substrates, it is necessary to evaluate the survival rate of Bael *in vitro*. To evaluate the viability, *in vitro* Bael were grown on sterilized coir. The results of the survivability survey are shown in Table 4 and Fig. 3.

Table 4
Living plant percentage on coconut coir

Time	Live plants (%)	Shoot/sample	Leaves	Height plant (cm)	Plant color	Growth ability
4 weeks	100	1.10 ± 0.31	3.23 ± 1.35	3.34 ± 1.78	healthy green	Good
6 weeks	100	1.10 ± 0.31	3.60 ± 1.41	3.74 ± 1.85	healthy green	Good
8 weeks	100	1.10 ± 0.31	4.32 ± 1.29	4.19 ± 1.99	healthy green	Good

Table 4 reveals promising results for coconut fiber as a substrate for Bael survival in net houses. After eight weeks of testing under these conditions, 100% of the *in vitro* Bael plants remained alive. While the average number of top shoots remained somewhat static at 1.10, the plants exhibited significant increases in both leaf count (4.32 leaves) and height (4.19 cm). Notably, the leaves and plants maintained a healthy green color, indicative of good seedling growth. These findings suggest successful adaptation of *in vitro* Bael to natural conditions, paving the way for its potential use in wild propagation efforts. Moreover, post-coir growth provided valuable insights into the Bael plants' growth potential. The results can be seen in Table 5 and Fig. 4.

Table 5
Effect of growing mediums to living plant of *in vitro* Bael

Growing mediums	Live plants (%)	Shoot/sample	Leaves	Height plants (cm)	Plants color	Growth ability
After eight weeks						
Formula 1	80	0.90 ± 0.57	4.20 ± 2.35	2.93 ± 1.82	healthy green	Good
Formula 2	**100**	**1.30 ± 0.48**	**4.77 ± 1.14**	**4.52 ± 2.93**	healthy green	Best
Formula 3	90	1.00 ± 0.47	3.30 ± 1.70	3.99 ± 2.21	healthy green	Good
After 10 weeks						
Formula 1	80	0.90 ± 0.57	4.20 ± 2.35	3.15 ± 1.92	healthy green	Good
Formula 2	**100**	**1.30 ± 0.48**	**4.70 ± 1.16**	**4.83 ± 2.92**	healthy green	Best
Formula 3	90	1.00 ± 0.47	3.30 ± 1.70	4.23 ± 2.29	healthy green	Good

The results in Table 5 showed that the survival rate of plants in the media after 10 weeks of planting ranged from 80% to 100%. On the formula 1, the survival rate was 80%, the plants had the worst growth, both the number of shoots (reaching 0.90 shoots) and the number of leaves (reaching 4.20 leaves). plant height (reaching 3.15 cm) was lower than in other treatments. For the formula 3, plants grow and develop normally. On the formula 2 (Table 5), the survival rate reached 100%, the tree was sturdy and grew well, healthy green leaves, thick and large leaf blades. Like other treatments, the number of shoots did not increase much, but the number of leaves (4.70 leaves) and plant length (4.83 cm) both increased and were better than other treatments. When growing out of the substrate, if the substrate is meat soil mixed with coconut fiber, it will increase the ability to absorb water and keep the soil moist, in addition, it will also increase the aeration of the soil to make the plant grow better. Therefore, in the recipe, the tree often mixes coconut fiber into it. Thus, within the scope of the experiment, the suitable medium for the growing stage in the nursery rice husk + coconut fiber + loam (ratio 1:2:2).

Limitations

This study has certain limitations that should be considered when interpreting the results. First, the number of explants and replicates was relatively modest, which may restrict the statistical robustness and generalizability of the findings. Second, only a limited set of auxin–cytokinin combinations was tested; additional concentrations or growth regulator types might further enhance shoot proliferation and rooting efficiency. Third, acclimatization trials were conducted under controlled greenhouse conditions,

and further field-based evaluations are needed to validate survival rates under variable environmental conditions. In addition, molecular and genetic stability of the regenerated plantlets was not assessed, and future studies should incorporate DNA-based markers to ensure clonal fidelity. Finally, the study focused primarily on morphological and physiological responses, without exploring biochemical or molecular mechanisms underlying the observed growth patterns. Despite these limitations, the results provide a valuable, reproducible protocol that contributes to the conservation and large-scale propagation of *Aegle marmelos*.

Abbreviations

BAP
6-benzylaminopurine
IBA
indole-3-butyric acid
IAA
indole-3-acetic acid
α-NAA
α-naphthaleneacetic acid
MS
Murashige and Skoog medium
ANOVA
Analysis of Variance
DMRT
Duncan's Multiple Range Test.

Declarations

Ethics approval and consent to participate

Permission for sample collection was granted by the Faculty of Biology, Thai Nguyen University of Education (Vietnam). The plant materials used in this study were not endangered or protected species.

Consent for publication

Not applicable.

Competing Interests

The authors declare that there are no competing interests.

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Author Contribution

HPH conceived and designed the study. TTH and HPH performed the experiments. TTH and HPH analyzed the data. HPH drafted the manuscript. All authors reviewed and approved the final version.

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Data Availability

All data supporting the findings of this study are included in the article.

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Figures

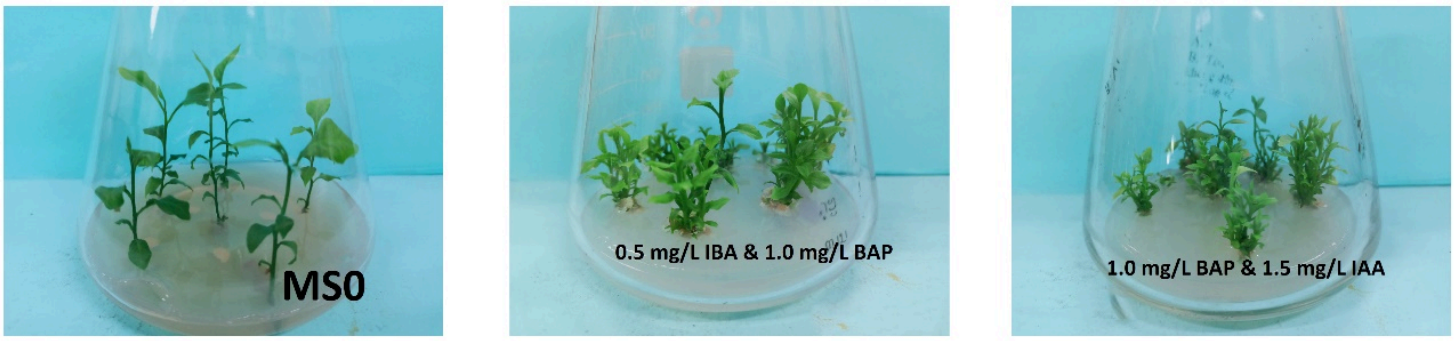


Figure 1

Growth of *in vitro* Bael shoots after eight weeks

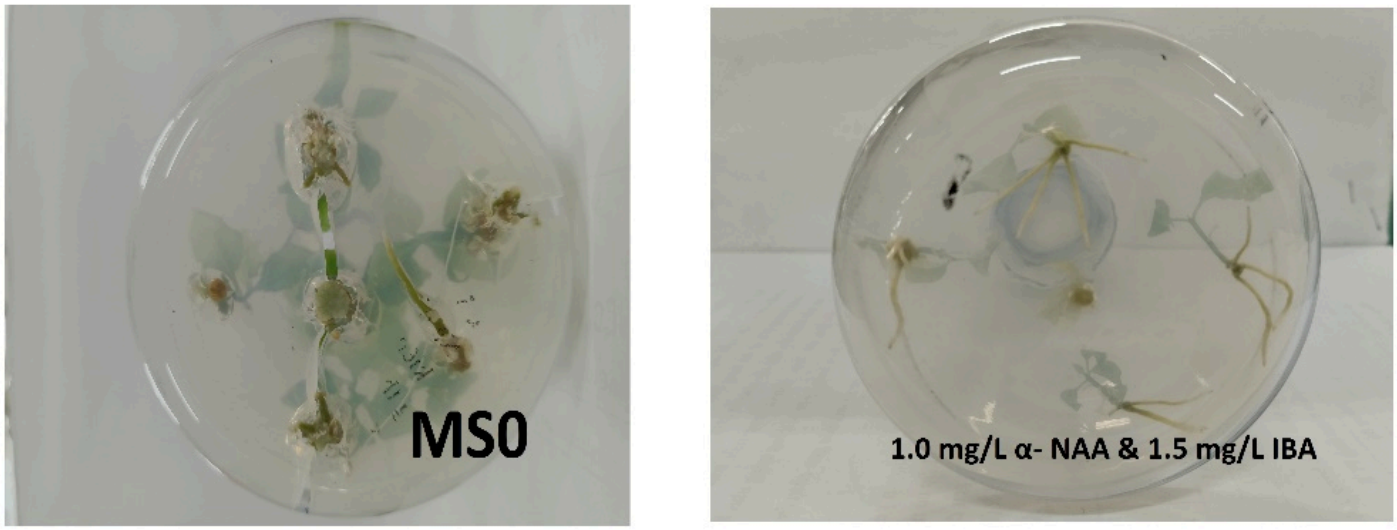


Figure 2

Roots of Bael *in vitro* after eight weeks of cultured on the medium with 1.0 mg/L α- NAA and 1.5 mg/L IBA



Figure 3

Image of *in vitro* Bael growing on coconut coir after eight weeks



Figure 4

Image of *in vitro* Bael growing after eight weeks