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# Potential Utilization of Coconut Water as a Natural Substitute to Plant Growth Regulators for *In Vitro* Propagation of *Hibiscus sabdariffa*

**Oluwakemi Adetutu Bello[1,2*], Adepeju Deborah Adedeji[1] and Olawole Odun Obembe[1,2,3]**
[1]*Department of Biological Sciences, Covenant University, Ota, Ogun State, Nigeria*
[2]*Plant Science Research Cluster, Covenant University, Ota, Ogun State, Nigeria*
[3]*UNESCO Chair on Plant Biotechnology*
[*]For correspondence: adetutu.bello@covenantuniversity.edu.ng
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## Abstract

*Hibiscus sabdariffa* L. possesses various parts like seeds, leaves and calyces that hold economic importance due to their multi-purpose. Its benefits can be fully tapped into using *in vitro* propagation, especially by incorporating organic additives into the media. This study established the sterilization protocol and the response of H. *sabdariffa* to various concentrations of coconut water as a natural plant growth regulator. Seeds were surface-sterilized using calcium hypochlorite solution in combination with 70% ethanol, with or without hot water pre-treatment, and cultured on Murashige and Skoog (MS) basal media. Ten days after culture, hypocotyl, cotyledon and cotyledonary node explants excised from *in vitro-grown* seedlings were cultured on MS medium supplemented with varying concentrations of coconut water (0, 5, 10, 15, 20 and 25% v/v). The cultures were monitored for callus induction, morphology, and colour of the callus, as well as shoot and root formation. The result showed that the seeds sterilized in 70% ethanol (20 s) and 5% calcium hypochlorite for 20 min resulted in the highest percentage of clean culture and germination. By day 28, all the explants in the different coconut water treatments had formed callus. Leaf formation was observed from all cotyledonary nodes in the various treatments. Multiple shoots were observed on cotyledon explants in MS medium supplemented with 15% coconut water. Root formation was observed on all the treatments. Further research is recommended to ascertain the effect of coconut water on the morphogenesis of the plant.

**Keywords:** Coconut water; Organic additives; *Hibiscus sabdariffa*; Roselle; Multiple shoots

## Introduction

Roselle (*Hibiscus sabdariffa* Linn.), a widely cultivated plant, has parts like seeds, leaves, and calyces that hold economic importance due to their multi-purpose applications (Chew *et al.* 2024). The seeds offer high phenolics, fibre and protein suitable for functional food development; seed oil provides a unique fatty acid composition beneficial for health, while leaves and calyces contribute valuable phenols, flavonoids, and anthocyanins for applications in nanoparticles, food, and colourant industries (Omole and Oranusi 2019; Emetere 2020; Omole *et al.* 2022; Chew *et al.* 2024).

It is important to develop methods of rapidly supplying clean and genetically homogenous planting material of roselle to farmers for stress-free planting (Govinden-Soulange *et al.* 2009). This is achievable by *in vitro* propagation and is also important for the conservation of its genetic resources (Manokari *et al.* 2016). Several literatures on the *in vitro* propagation of *H. sabdariffa* have been published. Shoots have been induced using shoot apex explants on Murashige and Skoog (MS) medium supplemented with 17.74 $\mu M$ 6-benzyladenine (BA), thidiazuron and m-topolin (Gomez-Leyva *et al.* 2008), nodal explants from *in vitro* seedlings on MS basal medium supplemented with 2.0 mg $L^{-1}$ 6-benzylaminopurine (BAP), 0.5 mg $L^{-1}$ indole-3-acetic acid (IAA), and either 10 $\mu M$ silver nitrate ($AgNO_3$) or 20 $\mu$g $L^{-1}$ triacontanol (TRIA) (Kumar *et al.* 2016). Leaf, node, and internodal explants also induced calli (friable/compact) in a media combination of 2,4-D, NAA and BAP (Raaman *et al.* 2013).

Mineral compositions as well as growth regulators are crucial components of the *in vitro* culture medium (Peixe *et al.* 2007). Growth regulators (hormones) are the most expensive of all the chemicals used in plant tissue culture, though they are needed in minute quantities in the medium (Prakash *et al.* 2004). Meanwhile, in developing countries, the unit production cost of micropropagated plants is high, affecting the return on investment. Therefore, the use of low-cost alternatives can greatly reduce the cost of tissue culture (Naik *et al.* 2020). The inclusion of organic additives

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such as malt extract and coconut water, banana, and papaya fruit juices, which provide indeterminate blends of organic nutrients and growth factors in culture media, has been found to provide a cost-effective option as a plant growth stimulant for *in vitro* plant regeneration (Wong *et al.* 2024). Specifically, coconut water, the liquid endosperm of *Cocos nucifera* L. is usually discarded by consumers who eat only the meat (Ng *et al.* 2020), but it composes of sugars, amino acids, organic acids, vitamins, alcohol sugar, lipids and N-*dhiphenyl urea* - unctioned as auxins and cytokinins (Yong *et al.* 2009). It is affordable, easily accessible, simple to prepare and used in the plant growth medium (Klanrit *et al.* 2023) to enhance callus induction and proliferation (Kamaruzzaman *et al.* 2018).

The use of coconut water as a supplement in *in vitro* propagation of economically important species has been studied by various scientists with varying results. They are added either to the basal media or used to replace them. They can also be used alone or in combination with synthetic plant growth hormones. Shoot induction and elongation, root induction and elongation, and leaf formation in *Irvingia gabonensis* (Gbadamosi and Sulaiman 2012); callus initiation, proliferation and regeneration potential in sweet potato (Michael 2012); shoot elongation in *Hylocereus polyrhizus* (Ng *et al.* 2020); multiple shoots and shoot length in *Ficus carica* cv. VDS regeneration (Lui *et al.* 2022); wider leaves, sturdier stems, shoot multiplication in strawberry (Kaur *et al.* 2022); shoot multiplication and root development in *Ensete glaucum* (Vu *et al.* 2023); shoot and root formation in *Asparagus officinalis* (Klanrit *et al.* 2023); shoot length, leaf count, and root length in sweet kaffir lime (Handayani *et al.* 2024). Coconut water induced callus (Sophia *et al.* 2021), shoot formation (Sophia *et al.* 2021; Figiel-Kroczyńska *et al.* 2022; Karunarathna *et al.* 2022; Lee *et al.* 2022), shoot height (Ng *et al.* 2020; Sophia *et al.* 2021; Figiel-Kroczyńska *et al.* 2022; Lee *et al.* 2022) and number of leaves (Sophia *et al.* 2021; Karunarathna *et al.* 2022; Lee *et al.* 2022) in several plant species.

The use of coconut water rather than laboratory-synthesized plant growth hormones can reduce the cost of tissue culture and save resources needed for the purchase of these plant hormones. The aim of this study was to explore the optimal surface sterilization regime for culture establishment, as well as examine the impact of varying coconut water concentrations and *in vitro-grown* explant types on callus formation and organogenesis of H. *sabdariffa in vitro* propagation.

## Materials and Methods

### Source of experimental material

Seeds of *H. sabdariffa* var. *sabdariffa* were requested for and obtained from the seed Genebank of the National Centre for Genetic Resources and Biotechnology (NACGRAB), Ibadan, Nigeria. The research study was carried out in the Plant tissue culture laboratory of the Department of Biological Sciences, College of Science and Technology, Covenant University, Ota, Ogun State, Nigeria. Culturing and sub culturing of explants were all done under aseptic conditions. All tools and media used in the study were sterilized appropriately. Ethanol (70% v/v) was used to sterilize the laminar flow hood and hands of the operators.

### Surface sterilization

Double sterilization protocol using 70% ethanol and calcium hypochlorite ($Ca(ClO)_2$) at varied concentrations and periods was employed in this study to surface-sterilize the seeds. The seeds in all treatments were washed with liquid soap and rinsed under running tap water to remove any dirt on their surfaces. After washing, the seeds were exposed to the different treatments (Table 1). However, the seeds in treatments SST1 – SST4 were pre-treated with hot water. After surface sterilization, excess water on the seeds was dried by placing the seeds on sterile filter paper.

### Seed germination and growth condition

The previously prepared culture media used for the initiation of seeds was semi-solid Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) with a pH adjusted to 5.7 using 1 *M* NaOH/HCl and then solidified with 7 g $L^{-1}$ agar as the gelling agent. The media was sterilized using the autoclave at 121ºC for 15 min, was allowed to cool, and observed for at least 3 days for non-contamination of the media.

The sterilized seeds were placed carefully into the media. Caution was taken not to submerge the seed into the media. The culture tubes were covered and sealed with parafilm. The cultures were kept in the growth chamber at a temperature of 25 ± 2ºC and a photoperiod of 16/8 h. They were observed for 2 weeks to document the cleanliness of the culture and seed germination.

### Preparation of media with coconut water

The ideal concentration of coconut water for a plant species must be determined through experimentation (Al-Khayri 2010).

The coconut water used in this study was prepared from mature coconut fruits. A sharp object was used to drill holes through the two of the micropyles. After collecting the coconut water, it was filtered with 4 layers of cheesecloth to remove particles and then heated at 80ºC for 10 min. As a result of heating, a precipitate formed, and the coconut water was then filtered with a filter paper to obtain a clear and slightly yellow liquid, which was stored at -20°C (Thorpe *et al.* 2008). The frozen coconut water was totally thawed at subsequent uses. To induce *in vitro* responses, Al-Khayri (2010) recommended that coconut water be added to

the basal medium at concentrations between 5 and 30%. Therefore, semi-solid Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) was supplemented with different concentrations of coconut water (Table 2), pH adjusted to 5.7 using 1 *M* NaOH/HCl, and then solidified with 7 g $L^{-1}$ agar as the gelling agent. HSC (MS media without coconut water) was used as a control for the experiment. All the media were sterilized using the autoclave at 121ºC for 15 min, allowed to cool, and observed for at least 3 days for non-contamination of the media.

### *In vitro* morphogenesis in MS media and coconut water

Hypocotyl, cotyledon and cotyledonary node explants excised from 2-week-old *in vitro-grown* seedlings of *H. sabdariffa* were placed on the media for morphogenic responses. The hypocotyl explants were positioned using 2 orientations: vertical and horizontal.

### Statistical analysis

Statistical analysis of the result was done using GraphPad Prism 5. Descriptive statistics of the data showing the mean and standard error were done and represented in tables for all the treatments and explants.

## Results

### Cleanliness of culture and seed germination

For the initiation of this study, ethanol (70%) and different concentrations of calcium hypochlorite ($Ca(ClO)_2$) were used to sterilize seeds of *H. sabdariffa*. After 10 days in culture, SST5 (70% Ethanol for 20 s and 5% $Ca(ClO)_2$ for 20 min) resulted in 92% clean culture and 12% germination (Fig. 1).

### Callus induction/formation

In the experiment containing coconut water at varied concentrations in MS basal medium, it was observed that the different concentrations of coconut water were capable of inducing callus on all explants of *H. sabdariffa* after a 4-week culture period. Callus was observed around the cut ends of the cotyledon explants, and the surface of the hypocotyl explants placed horizontally on the medium across all treatments. For the cotyledonary node and hypocotyl placed vertically, calli were observed on the portion in direct contact with the medium. The first signs of callus formation included swelling of the explant and the emergence of white granules with a crumbly texture from the cut-end of the explants. These granules solidified over time. Subsequently, the irregular granules transitioned in colour to light green. Some callus observed was friable while others were compact. The callus observed at higher levels of

**Table 1:** Surface sterilization treatment for seed

| Treatment code | Treatments |
|---|---|
| SST1 | 70% Ethanol for 5 mints + 1% $Ca(ClO)_2$ for 10 mints |
| SST2 | 70% Ethanol for 5 mints + 2% $Ca(ClO)_2$ for 10 mints |
| SST3 | 70% Ethanol for 5 mints + 5% $Ca(ClO)_2$ for 10 mints |
| SST4 | 70% Ethanol for 5 mints + 10% $Ca(ClO)_2$ for 20 mints |
| SST5 | 70% Ethanol for 20 s + 5% $Ca(ClO)_2$ for 20 mints |
| SST6 | 70% Ethanol for 60 s + 5% $Ca(ClO)_2$ for 20 mints |
| SST7 | 70% Ethanol for 2 mints + 5% $Ca(ClO)_2$ for 20 mints |
| SST8 | 70% Ethanol for 5 mints + 5% $Ca(ClO)_2$ for 20 mints |

**Table 2:** Media constituents for coconut water treatment for *in vitro* culture of *H. sabdariffa*

| Media code | Treatments |
|---|---|
| HSC | MS + 0% coconut water |
| HS01 | MS + 5% coconut water |
| HS02 | MS + 10% coconut water |
| HS03 | MS + 15% coconut water |
| HS04 | MS + 20% coconut water |
| HS05 | MS + 25% coconut water |

**Table 3:** Effect of coconut water on percentage callogenic response of *H. sabdariffa* after 28 days

| Treatment | Hypocotyl (vertical) (%) | Hypocotyl (horizontal) (%) | Cotyledon (%) | Cotyledonary node (%) |
|---|---|---|---|---|
| HSC | ++++ | ++++ | – | – |
| HS01 | ++++ | ++++ | ++++ | ++++ |
| HS02 | ++++ | ++++ | ++++ | ++++ |
| HS03 | ++++ | ++++ | ++++ | ++++ |
| HS04 | ++++ | ++++ | ++++ | ++++ |
| HS05 | ++++ | ++++ | ++++ | ++++ |

-= no response; += 0-25%, ++ = 26-50%, +++ = 51-75%, ++++ = 76-100%

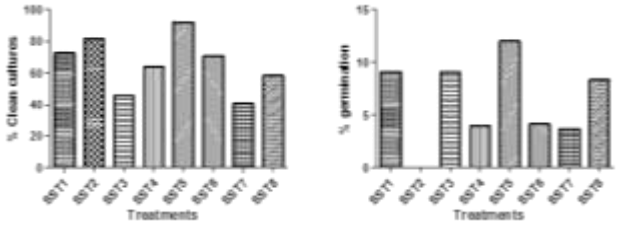


**Fig. 1:** Effect of surface sterilization regimes on percentage clean culture and germination of *H. sabdariffa* seeds after 10 days

coconut water was more compact and denser than those at lower concentrations (Table 3). The callus induction on the hypocotyl placed horizontally was greater than that on the cotyledons (Fig. 2A, B).

### Number of leaves

The media supplemented with 5 and 20% coconut water (HS01 and HS04) were optimal for the highest number of leaves (4.00 ± 0.00) in the cotyledonary node. Only 15% coconut water (HS03) induced leaves (10 leaves) in cotyledon explant. The hypocotyl did not initiate shoot regeneration at both orientations (vertical and horizontal) in all treatments. Meanwhile, the control (media not supplemented with coconut water) produced a node per shoot (1.00 ± 1.00) (Table 4).

**Table 4:** Number of leaves/nodes of *H. sabdariffa* in coconut water treatment after 4 weeks (28 days)

| Treatment | Age of explant (days) | Hypocotyl (vertical) | Hypocotyl (horizontal) | Cotyledon | Cotyledonary node |
|---|---|---|---|---|---|
| HSC | 28 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 1.00 ± 1.00 |
| HS01 | 28 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 4.00 ± 0.00 |
| HS02 | 28 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 3.00 ± 1.00 |
| HS03 | 28 | 0.00 ± 0.00 | 0.00 ± 0.00 | 10.00 ± 0.00 | 3.50 ± 1.50 |
| HS04 | 28 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 4.00 ± 0.00 |
| HS05 | 28 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 3.50 ± 1.50 |

Results expressed in mean ± standard error

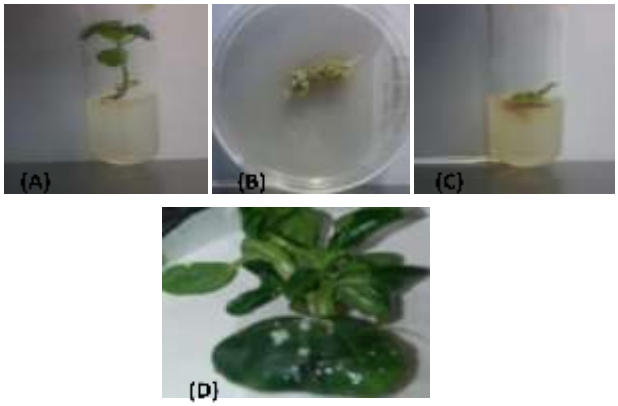


**Fig. 2:** Development of explants after *in vitro* culture in different coconut water treatment (**A**) Leaf formation and root formation from cotyledonary node of *H. sabdariffa* in MS medium supplemented with 5% coconut water (HS01) at 28 days. (**B**) Callus induction on hypocotyl placed horizontally in MS medium supplemented with 25% coconut water (HS05) at 28 days. (**C**) Callus induction on hypocotyl placed vertically in MS medium supplemented with 25% coconut water (HS05) at 28 days. (**D**) Multiple shooting and leaf formation on Cotyledon in MS medium supplemented with 15% coconut water (HS03)

**Shoot formation**

A single shoot was observed in all the cotyledonary node explants placed in the medium with various concentrations of coconut water at 4 weeks after culture. Meanwhile, the hypocotyl did not initiate shoot regeneration at both orientations (vertical and horizontal) in all treatments. However, the medium supplemented with 15% coconut water (HS03) induced the greatest proliferation of multiple shoots (3.33 ± 3.33) at the cut end of the cotyledon explants (Table 4 and Fig. 2C, D).

**Root induction/formation**

Root formation was observed in all the different coconut water treatments but only on some explants. Only HS04 and HSC induced root on the cotyledons (Table 5). In explants that had callus, the root began to grow from the callus, which depicts indirect organogenesis. The cotyledons in the control medium gave the longest root (Table 6).

## Discussion

After a 4-week culture period, it was observed that the various concentrations of coconut water used were capable of inducing callus on all explants of *H. sabdariffa*. The callus initiation potential of Roselle was reported by Abeda *et al.* (2014) but a greater callus on the cotyledon. Callus was observed around the cut ends of the cotyledon explants, and the surface of the hypocotyl explants placed horizontally on the medium across all treatments. For the cotyledonary node and hypocotyl placed vertically, calli were observed on the portion in direct contact with the medium. This agrees with the report of Markin *et al.* (2023) that injuries during the excision of explants from six cowpea accessions led to callus formation alongside root growth when cultured on MS media supplemented with coconut juice. The transitioning of callus colour to light green agrees with Andaryani *et al.* (2019), which also suggests that the colour of a callus may reflect the amount of chlorophyll it contains, stating that a greener callus signifies higher chlorophyll content. Also, Kresnawati (2006) stated that the green and white colours of the callus suggest that it is still in good condition. The highest callus formation rate (50%) was also reported with 100 mL $L^{-1}$ and 300 mL $L^{-1}$ coconut water in *Ficus Carica* (Sophia *et al.* 2021).

MS basal media supplemented with coconut water enhanced plant height potentially due to increased gibberellin concentrations and root development attributed to higher auxin levels in the extracts (Markin *et al.* 2023). This result agrees with several reports on the effect of coconut water alone or combined with synthetic plant growth hormones on the number of leaves in different plants. The highest number of leaves (5.80) was achieved

**Table 5:** Percentage root formation of *H. sabdariffa* in coconut water treatment four week after culture

| Treatment | Hypocotyl (vertical) (%) | Hypocotyl (horizontal) (%) | Cotyledon (%) | Cotyledonary node (%) |
|---|---|---|---|---|
| HSC | ++ | – | ++++ | – |
| HS01 | + | ++++ | – | ++++ |
| HS02 | – | ++ | – | – |
| HS03 | – | ++ | – | ++ |
| HS04 | – | – | ++ | – |
| HS05 | – | + | – | – |

-= no response; += 0-25%; ++ = 26-50%; +++ = 51-75%; ++++ = 76-100%

**Table 6:** Root length (in cm) of *H. sabdariffa* in coconut water treatment Week 4 (28 days) after culture

| Treatment | Hypocotyl (vertical) | Hypocotyl (horizontal) | Cotyledon | Cotyledonary node |
|---|---|---|---|---|
| HSC | 7.70 ± 7.70 | 0.00 ± 0.00 | 13.55 ± 12.85 | 0.00 ± 0.00 |
| HS01 | 0.60 ± 0.60 | 10.37 ± 3.91 | 0.00 ± 0.00 | 5.85 ± 0.25 |
| HS02 | 0.00 ± 0.00 | 1.38 ± 0.53 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| HS03 | 0.00 ± 0.00 | 0.80 ± 0.80 | 0.00 ± 0.00 | 4.90 ± 2.45 |
| HS04 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.47 ± 0.09 | 0.00 ± 0.00 |
| HS05 | 0.00 ± 0.00 | 0.43 ± 0.43 | 0.00 ± 0.00 | 0.00 ± 0.00 |

Results expressed in mean ± standard error

with 1 mg $L^{-1}$ BAP + 200 mL $L^{-1}$ CW (Sophia *et al.* 2021). Also, 200 mL $L^{-1}$ coconut water enhanced induction of leaves (5.08 ± 0.81 leaves per explant) in *Ficus carica* cv. Japanese BTM6 (Lee *et al.* 2022). Meanwhile, 30% CW with 0.2 mg $L^{-1}$ 6-benzylamino purine (BAP) resulted in the highest leaf production after three weeks, with an average of 8.28 ± 0.81 leaves per sample in *Stevia Rebaudiana* (Karunarathna *et al.* 2022). In another report, 30% concentration of coconut water enhanced leaf count in *in vitro* propagation of sweet kaffir lime (Handayani *et al.* 2024).

The single shoot observed showed the potential of the cotyledonary node to regenerate. This agrees with the report of Markin *et al.* (2023) that cotyledonary nodes are the most receptive to *in vitro* culture in cowpea. The kind of explant used has been reported to significantly influence shoot regeneration (Phillips and Hubstenberger 1985). Karunarathna *et al.* (2022) reported that coconut water by itself was not enough to achieve satisfactory shoot multiplication from the nodal explants of *Stevia rebaudiana*. The induction of multiple shoots (3.33 ± 3.33) at the cut end of the cotyledon explants (Table 4 and Fig. 2D) is likely due to the rich content of growth-promoting compounds in coconut water (Yong *et al.* 2009) and the regeneration potential of the explant. This result on the effectiveness of coconut water as an organic growth factor agrees with published reports. Medium supplemented with 200 mL $L^{-1}$ coconut water induced the highest number of shoots (3.03 ± 0.122) in *Ficus carica* cv. VDS regeneration (Lui *et al.* 2022). Coconut water, containing natural plant growth regulators, enhances shoot development in H. *costaricensis* explants (Norrizah *et al.* 2023). Apparently, adding a low concentration of coconut water improved shoot multiplication, whereas a higher concentration hindered growth (Karunarathna *et al.* 2022).

The root induced began to grow from the callus, which depicts indirect organogenesis. Injuries during the excision of explants from six cowpea accessions led to root growth when cultured on MS media supplemented with coconut juice (Markin *et al.* 2023). This could be due to the composition of coconut water, which includes thiamine and auxin that support the development of roots (Yong *et al.* 2009). The cotyledons in the control medium gave the longest root (Table 6). This agrees with the report of Sophia *et al.* (2021) that the greatest number of roots (17) was observed in the control group, while 9.5 roots were achieved with 200 mL $L^{-1}$ coconut water. MS medium with 5% coconut water also enhanced the longest root length (7.00 ± 0.66 cm) in H. *costaricensis* explants (Norrizah *et al.* 2023).

## Conclusion

Addition of 15% coconut water to basal media supported multiple shoots in cotyledon explant; 5% coconut water for root formation in hypocotyl (horizontal orientation) and cotyledonary node; as well as leaf formation in cotyledonary node explants of *H. sabdariffa*. Coconut water used in *in vitro* culture of *H. sabdariffa* at different concentrations also induced callus. Cost analysis and further research using other organic additives/ readily available alternatives can be done on different plants to establish cost-effective *in vitro* propagation in developing countries.

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## Author Contributions

Conceptualization: BOA and ADA; Methodology: BOA and ADA; Supervision: OOO and BOA; Writing – original draft: ADA; Writing – review and editing: BOA and OOO. All authors read and approved the final manuscript.

## Conflicts of Interest

The authors declare that there are no conflicts of interest related to this article.

## Data Availability

The corresponding author will provide the data from this study upon request.

## Ethics Approval

Not applicable to this paper.

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