Research Article

Comparison of Three Cytokinins on In vitro Multiplication of Orbea semota, a Conical, Stout-Teethed Succulent

Kemar A Rose¹, Collin M Scantlebury¹, Marsha C Williams¹, Ryan D Francis¹

¹Biotechnology Department, The Scientific Research Council, Kingston, Jamaica

Correspondence to: Kemar A Rose, MBA, BSc, Research Officer, Biotechnology Department, The Scientific Research Council, Hope Gardens, Kingston 6, Jamaica; Email: kemar.rose22@gmail.com

Received: December 25, 2023 Revised: May 5, 2024 Accepted: May 10, 2024 Published: May 16, 2024

Abstract

Objective: Orbea semota (N.E. Br.) L.C. Leach is an erect or procumbent succulent with numerous branches. The stems of this plant are grey-green to dark green in color and possess flowers that are dark maroon or dark brown with yellow marks at the tips. It is native to Kenya, Tanzania and Rwanda and grows in rocky places. There is great potential for its use as a medicinal plant, also, there are immense prospects for commercial trade and cultivation of the succulent as an ornamental in Jamaica. The purpose of the study is to select the most effective cytokinin for the shoot multiplication of Orbea semota using stem cuttings.

Methods: Young shoots were initiated for 6-8 weeks on a modified Murashige and Skoog (MS) medium. Initiated shoots were then cultivated on Media A, B, C and D for 8 weeks to promote shoot multiplication. These were modified MS media supplemented with three different cytokinins [6-γ, γ-Dimethylallylamino purine (2iP), 6-Benzylaminopurine (BA) and Kinetin] and a control without any cytokinins.

Results: Medium A (containing 2iP) produced the best results at shoot multiplication than all other media used. The mean lengths of the shoots produced by Medium A were 19.0±11.4 mm and 10.5±3.8 mm. The mean number of shoots generated using Medium A were 3.4±1.3 and 4.0±1.9. These results were significantly greater than those of the shoots produced by all other media.

Conclusion: The present study details an efficient and reproducible protocol for the in vitro shoot multiplication of Orbea semota. The results obtained showed that Medium A was the most optimal medium for promoting shoot multiplication. Therefore, of the three cytokinins investigated 2iP produced the best results at stimulating shoot multiplication and growth of Orbea semota.

Keywords: 2iP, Apocynaceae, Asclepiadoideae, in vitro shoot multiplication, Orbea semota
1 INTRODUCTION

*Orbea semota* (N.E. Br.) L.C. Leach, also called *Stapelia semota*, belongs to the subfamily *Asclepiadoideae* of the family Apocynaceae[1]. It is native to Kenya, Tanzania and Rwanda growing in rocky places between 800-2000m above sea level[2]. This plant is a much-branched succulent growing up to 10cm tall with erect or procumbent stems forming compact cushions. These 4-angled stems are 10-18mm across excluding their stout, conical teeth, which can ascend or spread to 12mm. The stems are typically grey-green or dark green, occasionally blotched with dark reddish-brown. The flowers are transversely wrinkled and can appear as solitary or several together borne from any region of the stem with a diameter of 3.5-5cm across; they are dark maroon or dark brown with yellow marks at the tips or throughout the lobes[3,4]. Due to its colorful flowers, this plant could be utilized as an ornamental in Jamaica. It also produces a foul-smelling odor, which is primarily caused by butanoic acid. There is potential for *Orbea semota* to be used as a medicinal plant as the sap from the pounded stems is used to treat wounds and ulcers in Tanzania[5].

*Orbea semota* can be propagated by using stem cuttings and seed sowing only in the spring[6]. However, there are challenges with traditional propagation techniques as rotting often occurs rather than rooting whenever *Asclepiadoideae* stem cuttings are planted upright in rooting substrate[7]. In light of the issues with the traditional methods of propagation and the immense potential for medical use, as well as commercial trade and cultivation as an ornamental, tissue culture propagation can be employed as a means to rectify both issues.

Plant tissue culture methods are conducted under controlled aseptic, nutritional and environmental conditions. These techniques produce plant tissues called explants which are indistinguishable replicas of the mother plant that are generated from the in vitro culture of cells, tissues, organs or the entire plant[8]. A single explant can be multiplied in a shorter time period compared to the traditional method and the demand for space is less; this is possible all throughout the year despite the season or weather[9].

In vitro morphogenesis is greatly impacted by plant growth regulators; cytokinins have been known as plant growth regulators since 1950 when their ability to stimulate cell division was discovered[10,11]. Therefore, cytokinins are generally used on micropropagation media to promote in vitro shoot multiplication[12].

To the best of our knowledge on propagation, no published study on the in vitro propagation of *Orbea semota* exists. Due to the medicinal and ornamental benefits of this plant, the Biotechnology Department of the Scientific Research Council is conducting research on this plant. Therefore, the objective of this study was to select the most effective cytokinin for the shoot growth and multiplication of *Orbea semota* stem cuttings.

2 MATERIAL AND METHODS

2.1 Plant Material and Initiation of Culture

*Orbea semota* plants were obtained locally and maintained in a shade-house under 75% shade at the Scientific Research Council, Kingston, Jamaica (18.0189° N latitude and 76.7497° W longitude) in November 2022. Young explant shoots 3-6mm long were washed with tap water and 1% detergent. The shoots were then treated in a fungicide and bactericide mixture of 2.5g/L 70% 1,2-di-(3-methoxycarbonyl-2-thioureido) benzene (thiophanate methyl) (Topsin-M 70% WP), 3g/L 4% Methyl N-(2,6-dimethylphenyl)-N-(methoxo acetyl) alaninate +64% Manganese ethylene bisdithiocarbamate (Ridomil 68 WP), 3mL/L 21.36% Copper Sulfate Pentahydrate (Phytogen-27 SC) for one hour by using an automatic stirrer to ensure continuous stirring.

Explants were surface sterilized by submerging shoots in a 70% ethanol solution for 1 minute, followed by 5.25% sodium hypochlorite plus Polyethylene glycol sorbitan monolaurate (TWEEN 80) for 15min. Disinfected explant shoots were rinsed in sterile distilled water 5-6 times.

The shoots were then cultured in Medium A since its protocol was used in the efficient in vitro propagation of another *Asclepiadoideae* species, *Edithicolea grandis*[13].

Medium A comprises of Murashige and Skoog (MS) basal salts[14], vitamins [100mg/L Myo-inositol (PhytoTechnology Laboratories, Shawnee Mission, Kansas, USA), 30mg/L Thiamine hydrochloride (HCl) (Sigma-Aldrich, St. Louis, Missouri, USA), 1mg/L Pyridoxine HCl (PhytoTech Labs, Lenexa, Kansas, USA), 10mg/L Nicotinic acid (PhytoTechnology Laboratories, Shawnee Mission, Kansas, USA), 10mg/L L. Arginine (PhytoTech Labs, Lenexa, Kansas, USA), 80mg/L Adenine sulfate (PhytoTech Labs, Lenexa, Kansas, USA), 10mg/L 6γ-γ-Dimethylallylamino purine (2iP) (Sigma Chemical Co., St. Louis, Missouri, USA), 0.5mg/L Indole-3-acetic acid (IAA) (Acros Organics, New Jersey, USA), 1mg/L Indole-3-butyric acid (IBA) (PhytoTech Labs, Lenexa, Kansas, USA), 45g/L sucrose and 2.5g/L PhytagTM (Sigma-Aldrich, St. Louis, Missouri, USA). The pH was adjusted to 5.3 and the medium was autoclaved at 121°C and 15 psi for 20min. Cultures were maintained for 6-8 weeks at 25±2°C with 16-h photoperiod under light emitting diode (LED) light with photon flux of 50 μmol m⁻² s⁻¹.

2.2 Shoot Multiplication

Shoots were cultivated on the same modified MS media supplemented with three different cytokinins. Medium A contained 10mg/L 2iP, medium B comprised 10mg/L 6-Benzylaminopurine (BA) (Beantown Chemical, Hudson, New Hampshire, USA) and medium C constituted 10mg/L Kinetin (Sigma Aldrich, St. Louis, Missouri, USA).
was no added cytokinin in Medium D (the control). In each treatment, 15 explants were used and after an 8-week incubation period, the mean shoot number generated per explant and the shoot lengths were determined. Each experiment was repeated.

2.3 Statistical Analysis

Analysis of variance (ANOVA) was used to estimate the effects of cytokinins and repetition of the experiment on shoot multiplication rate (number of shoots generated per explant) and shoot length. This followed two-way ANOVA methodology by Fowler et al. [15] where the total sum of squares for interaction (SS_i) and within sum of squares (SS_within) (Equation (1)).

$$SS_i = SS_a + SS_b + SS_i + SS_{within}$$ (1)

The Tukey’s Test in two-way ANOVA was used to determine significant differences between means (Equation (2)).

$$T = q \sqrt{\frac{within variance}{n}}$$ (2)

3 RESULTS

Figure 1A and B shows the in vitro propagation of Orbea semota explants. There were significant differences in explant shoot length between the four test media. There were also significant differences in shoot multiplication rates.

3.1 Shoot Length

Table 1 shows the differences in shoot length among media containing different cytokinins. For both subcultures, 2iP produced the greatest shoot length. The two-way ANOVA summary table (Table 2) indicated that while there was no difference in shoot length between subcultures, nor interaction, the difference in shoot length was significant at $P=0.01$, $F=419.079$ for $df_{3,112}$. The Tukey statistic indicated that the shoot length in Medium A (2iP supplemented medium) was greater than Medium B (BA supplemented medium); Medium B (BA supplemented medium) was greater than Medium C (kinetin supplemented medium) and the control.

3.2 Shoot Number Per Explant

Table 3 shows the differences in shoot number generated per explant among media containing different cytokinins. For both subcultures, 2iP generated the largest quantity of shoots per explant. The two-way ANOVA summary table (Table 4) indicated that while there was no difference in shoot number per explant between subcultures, nor interaction, the difference in number per explant was significant at $P=0.01$, $F=65.645$ for $df_{3,112}$. The Tukey statistic indicated that the quantity of shoots produced in Medium A (2iP supplemented medium) was greater than...
Table 3. Effect of Media Containing Different Cytokinins on Number of Shoots Generated per Explant During Multiplication of *Orbea Semota* After 8 Weeks of Culture

<table>
<thead>
<tr>
<th>Media (Cytokinin)</th>
<th>Number of Shoots Subculture 1</th>
<th>Number of Shoots Subculture 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (2iP)</td>
<td>3.4±1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.0±1.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>B (BA)</td>
<td>2.7±1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.3±1.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C (Kinetin)</td>
<td>1.5±0.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.9±1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>D/control (no cytokinin)</td>
<td>1.4±0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.8±1.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Notes: Significant differences were determined by the Tukey’s test and are indicated by superscripts with different letters (P≤0.01). Values are mean±SD. n=15.

Table 4. ANOVA Summary Table for Shoot Number per Explant Data

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Variance</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Between samples)</td>
<td>(11.927)</td>
<td>(7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcultures</td>
<td>0.002</td>
<td>1</td>
<td>0.002</td>
<td>0.028</td>
</tr>
<tr>
<td>Media (Cytokinins)</td>
<td>11.725</td>
<td>3</td>
<td>3.908</td>
<td>64.64&lt;sup&gt;−&lt;/sup&gt;</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.201</td>
<td>3</td>
<td>0.067</td>
<td>1.106</td>
</tr>
<tr>
<td>Within samples</td>
<td>6.771</td>
<td>112</td>
<td>0.060</td>
<td></td>
</tr>
</tbody>
</table>

Notes: <sup>−</sup> Indicates significance at P=0.01.

Medium B (BA supplemented medium); Medium B (BA supplemented medium) was greater than Medium C (kinetin supplemented medium) and the control.

4 DISCUSSION

There is great potential for *Orbea semota* as a medicinal plant as the stems of the *Orbea* genus have been used for a variety of medicinal purposes including the treatment of burns, eczema, wounds, diabetes, constipation, stomach ulcers and as an antidote for food poisoning<sup>[16]</sup>. The stems and roots of these plants have also been utilized in the suppression or curbing of appetite and the treatment or prevention of obesity<sup>[17]</sup>. Al-Fatimi et al<sup>[18]</sup> and Mubarak et al<sup>[19]</sup> have also reported on the antibacterial and cytotoxic effects of the *Orbea* genus. *Orbea semota* is a succulent that also has considerable prospects as an ornamental. The *Orbea* genus has high horticultural value and is in high demand by succulent collectors; plants belonging to this genus are grown and exchanged globally. The flowers are beautiful and their purple blotchy stems against different shades of green backgrounds tend to be extremely attractive<sup>[20]</sup>. The plants can be grown in large pots, in gardens or in rockeries<sup>[21,22]</sup>. The practical applications of our findings can be of particular interest to stakeholders within the agricultural and horticultural sectors. The utilization of plant tissue culture allows for optimization in growth conditions as *Orbea semota* plantlets will have an avenue in which they can be cultivated all year round, even when weather and climate conditions become unfavorable. Additionally, yield can be maximized using the plant tissue culture technique as this method of propagation has a higher multiplication rate than traditional methods.

Surface sterilants can be described as chemicals that eliminate or reduce various pathogens, including fungal spores, from plants. Usually, surface sterilants act on the outer portions of the explants. Many chemicals have been used to free plants from pathogens prior to culture initiation onto the media (chemotherapy). They inhibit the growth and proliferation of pathogens (fungi, bacteria and other kinds of microorganisms). To improve the efficacy of the surface cleaning of stock plants, many surface sterilants, disinfectants, and/or fungicide combinations are used subsequent to washing with water. Additionally, rinsing the stock plants in water may enhance the penetration of fungicides into the outer plant cells. In general, soapy water or detergent has been widely used in many plant tissue culture laboratories to enhance the elimination of pathogens and/or fungal spores from the stock plants. Ethanol is another commonly used disinfectant in plant culture laboratories; it is used to reduce culture contaminants by killing fungal pathogens and spores in stock plants. To decrease the deleterious impact of surface sterilants used to remove contaminants from plants, copious amounts of water must be used to rinse stock plants as this helps halt the reaction between the chemicals and the stock plants<sup>[24,25]</sup>. Previous reports have demonstrated the effectiveness of fungicides and bactericides in the reduction of contaminants during the *in vitro* propagation of other plant species. Pre-treatments with these chemicals were able to significantly decrease contamination levels<sup>[26,27]</sup>. The fungicides/bactericides used in this present study were Topsis-M 70% WP, Ridomil 68 WP and Phyton-27 SC. It has been established that common sterilizing agents such as sodium hypochlorite (5%-10%) and ethanol (50%-95%) can be used to exclude the surface contaminants by washing in the appropriate solution for 10-25min; surfactants are often added to sterilizing solutions to enhance sterilization techniques. Ethanol is a potent sterilizing agent; however, it is extremely phytotoxic. Hence, the explant is generally exposed to it for a few seconds or minutes<sup>[28]</sup>. In this present study, explants were surface sterilized using 70% ethanol for one minute and 5.25% sodium hypochlorite (with a few drops of the surfactant, Tween 80) for 15min to minimize contaminants.

Environmental factors such as light conditions and temperature can significantly affect *in vitro* cultures. Artificial light, in combination with other factors such as medium composition, gas exchange in the culture vessel and temperature, plays a critical role in successful *in vitro* plant production. Maintaining the appropriate photon flux and photoperiod enable the production of plants with desired traits<sup>[29]</sup>. If the photon flux is too low, this may negatively
Innovation Forever Publishing Group Limited

In plants, cytokinins are a category of phytohormones derived from adenine that stimulate cell differentiation. Cytokinin signaling is initiated by membrane-associated histidine kinase receptors and transduced through a phosphorelay system. The physiological function of cytokinins is to activate RNA, protein synthesis, and enzyme activity. Different cytokinins can also cause multiple shoot proliferation by interfering with shoot apical dominance. The multiplication of shoots is dependent on the accompanying of two iterative processes: the induction and formation of phytomer, which includes lateral meristem (axillary bud) formation from the apical meristem and the ensuing outgrowth of the axillary buds into newly developed shoots. Cytokinins are associated with the maintenance of shoot meristem function since they are positive regulators of stem meristem activity. Therefore, they play a crucial role in shoot development, especially, in the shoot apical meristem. There is an antagonistic relationship between cytokinins and auxins as cytokinin signaling is inhibited by auxin action in most parts of the plant. However, Ganesan and Jayabalai reported that high concentrations of cytokinins, in combination with low concentrations of auxins have been utilized for direct induction and elongation of multiple shoots in many plant species. This corresponds with the protocol used in this study as high concentrations of 2iP or BA (10mg/L), in combination with low concentrations of the auxins, IAA (0.5mg/L) and IBA (1mg/L) were able to induce shoot multiplication in Orbea semota. However, shoot multiplication produced by the cytokinin kinetin was not significantly different from the control. This is possibly due to kinetin being a weak cytokinin (especially when compared to BA) with low effectiveness in generating high shoot multiplication in some plant species. BA is the most commonly used cytokinin in commercial micropropagation, due to its efficacy and affordability. Other researchers of Apocynaceae species had optimal success in shoot multiplication with the use of kinetin, 2iP or BA. In addition, kinetin, 2iP and BA have been previously noted as some of the cytokinins that produced significant shooting response. Therefore, these three cytokinins were selected for this present study. The cytokinins present in Media A, B and C were 2iP, BA and Kinetin respectively while Medium D had no cytokinins present.

Medium A, containing 2iP, produced the greatest number of shoots (3.4±1.3 and 4.0±1.9) of all the media investigated, as well as generating the shoots with the highest mean shoot length (19.0±11.4mm and 10.5±3.8mm). Similar results were reported in Grewia tonax (Forsch.) Fiori and another member of the Apocynaceae family, Decalepis hamiltonii Wight & Arn. in which 2iP induced the greatest shoot multiplication of all three cytokinins. Kozak et al. also reported that 2iP produced better shoot multiplication than BA in Mandevilla sanderi (Hemsl.) Woodson, another Apocynaceae species. Contrary to the present findings, it was reported that in other members of the Apocynaceae family, Rauvolfia serpentina Benth. ex Kurz and Decalepis arayalpathra BA generated the best results for optimal shoot multiplication. Krishnareddy and Pullaiah and Muthukrishnan et al. reported that kinetin stimulated the greatest shoot multiplication in Ceropgia elegans and Ceropgia thwaitesii Hook. According to Enkhbileg et al., the difference in results could be due to the effects of cytokinins differing depending on the plant species.

There has been very little study on the micropropagation of the Orbea genus, so little is known about how cytokinins affect these plants. This present investigation provides a foundation for the in vitro propagation of plants within the Orbea genus. Since this protocol was also used in the efficient in vitro propagation of Edithcolea grandis, it may also provide a framework for the micropropagation of other species within the Asclepiadoideae subfamily that possess similar morphological structures.

https://doi.org/10.53964/jmab.2024003
5 CONCLUSION

In conclusion, the present study details an efficient and reproducible protocol for the in vitro shoot multiplication of *Orbea semota*. This current investigation assured effective establishment, mass multiplication and could offer an in vitro strategy for the ex situ conservation of this succulent that has great potential as a medicinal plant, as well as, commercial cultivation and trade as an ornamental in Jamaica. The results obtained showed that Media A was the most optimal medium at promoting shoot multiplication. Therefore, of the three cytokinins investigated 2iP produced the best results at stimulating shoot multiplication and growth of *Orbea semota*.

Acknowledgements

Support for this investigation was given by the Scientific Research Council, Jamaica. The authors wish to thank all our colleagues who provided their expertise that greatly assisted this research project and enhanced the manuscript.

Conflicts of Interest

The authors declared that they have no conflicts of interest.

Author Contributions

Rose KA conceived the study, designed the study, carried out the experimental work of the manuscript, generated the data, participated in the analysis and interpretation of the data and wrote the manuscript (including revisions). Scantlebury CM participated in the analysis and interpretation of the data and provided general supervision of the research. Williams MC shared in the research proposal and design of the study. Francis RD shared in the research proposal and provided general supervision of the research. All authors read and approved the final manuscript.

Abbreviation List

- 2iP, 6-γ, γ-Dimethylallylamino purine
- ANOVA, Analysis of variance
- BA, 6-Benzylaminopurine
- HCl, Hydrochloride
- IAA, Indole-3-acetic acid
- IBA, Indole-3-butyric acid
- LED, Light emitting diode
- MS, Murashige and Skoog

References


September 2006.


https://doi.org/10.53964/jmab.2024003