Research Square

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

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Research Article

Keywords: Orbea semota, In vitro micropropagation, 6-(γ, γ-Dimethylallylamino)Purine, Asclepiadoideae, shoot multiplication, cytokinin, Apocynaceae, succulent

Posted Date: December 4th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3660950/v1

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Abstract

Background

Orbea semota is a much-branched succulent with erect or procumbent grey-green to dark green stems with dark maroon or dark brown flowers possessing yellow marks at the tips. It is native to Kenya, Tanzania and Rwanda growing in rocky places and there is great potential for its use as a medicinal plant, as well as, commercial trade and cultivation as an ornamental in Jamaica. The purpose of the study is to select the most effective cytokinin for the shoot proliferation of *Orbea semota* using stem cuttings.

Results

Medium A (containing 2iP) produced the best results at shoot proliferation than all other media used. The mean lengths of the shoots produced by Medium A (19.0 ± 11.4 mm and 10.5 ± 3.8 mm) were significantly greater than that of the shoots produced by all other media. Significantly larger quantities of shoots were generated using Medium A than all other media (3.4 ± 1.3 and 4.0 ± 1.9).

Conclusions

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 Medium A was the most optimal medium at promoting shoot proliferation. Therefore, of the three cytokinins investigated 2iP produced the best results at stimulating shoot multiplication and growth of *Orbea semota*.

Background

Orbea semota, also called *Stapelia semota*, belongs to the subfamily Asclepiadoideae of the family Apocynaceae. It is native to Kenya, Tanzania and Rwanda growing in rocky places between 800 – 200 metres above sea level (Goyder et al. 2012). 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 (Fig. 1). 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 (Albers and Meve 2004; Cullen et al. 2011). Due to its colourful flowers, this plant could be utilized as an ornamental in Jamaica. It also produces a foul-smelling odour, 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 (Schmelzer and Gurib-Fakim 2013).

Orbea semota can be propagated by using stem cuttings and seed sowing only in the spring (Anonymous n.d.). 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 (Hodgkiss 2023). 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 (Rodge and Patil 2020). 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 (Suresh Kumar and Keshamma 2022).

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 (Nowakowska et al. 2022). Consequently, different cytokinins can also cause multiple shoot proliferation by interfering with shoot apical dominance (Ahmed 2022).

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.

Methods

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, 76.7497° W) in November 2022. Young explant shoots 3–6 mm 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-(methoxyacetyl) alaninate + 64% Manganese ethylenebisdithiocarbamate (Ridomil 68 WP), 3ml/L 21.36% Copper Sulfate Pentahydrate (Phyton-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 Polyethyelene glycol sorbitan monolaurate (Tween 80) for 15 minutes. Disinfected explant shoots were rinsed in sterile distilled water 5–6 times.

The shoots were cultured in modified Murashige and Skoog (MS) (1962) media previously used in the *in vitro* propagation of another Asclepiadoideae species, *Edithcolea grandis* (Rose et al. 2023). The pH was adjusted to 5.3 and the medium was autoclaved at 121°C and 15 psi for 20 minutes. Cultures were maintained for 6–8 weeks at 25 ± 2°C with 16-h photoperiod under fluorescent light with photon flux of 52 µmol m^{-2} s^{-1}.

Shoot proliferation

Shoots were cultivated on the same modified MS media supplemented with three different cytokinins. Medium A contained 10 mg/L 6-γ, γ-Dimethylallylamino purine (2iP), medium B comprised 10mg/L 6-Benzylaminopurine (BA) and medium C constituted 10mg/L Kinetin. There 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.

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. (1998) where the total sum of squares (SS_T) was partitioned into sum of squares for replications (SS_A), sums of squares of cytokinins (SS_B), sum of squares for interaction (SS_i) and within sum of squares (SS_{within}):

$SS_T = (SS_A + SS_B + SS_i) + SS_{within}$.

The Tukey Test in two-way ANOVA was used to determine significant differences between means.

$T = (q) \times \sqrt{(\text{within variance}/n)}$

Results

Figure 2a-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.

Shoot length

Table 1 showed the differences in shoot length among media containing different cytokinins. For both subcultures, 2iP produced the greatest shoot length (**See** Fig. 3). 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.

Table 1
Effect of media containing different cytokinins on shoot length during multiplication of *Orbea semota* after 8 weeks of culture.

Media (cytokinin)	Shoot length (mm)	
	Subculture 1	Subculture 2
A (2iP)	19.0 ± 11.4^{a}	10.5 ± 3.8^{a}
B (BA)	6.9 ± 4.1^{b}	6.4 ± 3.4^{b}
C (Kinetin)	5.5 ± 2.0^{c}	6.1 ± 2.1^{c}
D/control (no cytokinin)	6.2 ± 3.3^{c}	6.4 ± 3.0^{c}

Significant differences are indicated by superscripts with different letters ($p \leq 0.01$). Values are mean ± SD. n = 15

Table 2
ANOVA Summary Table for Shoot Length

Source of Variation	Sum of squares	df	Variance	F
(Between samples)	(83.756)	(7)		
Subcultures	0.004	1	0.004	0.055
Media (Cytokinins)	83.469	3	27.823	419.079**
Interaction	0.324	3	0.108	1.625
Within samples	7.436	112	0.066	
** indicates significance at P = 0.01				

Shoot number per explant

Table 3 showed 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 (**See** Fig. 4). 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 Medium B (BA supplemented medium); Medium B (BA supplemented medium) was greater than Medium C (kinetin supplemented medium) and the control.

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.

Media (cytokinin)	Number of shoots	
	Subculture 1	Subculture 2
A (2iP)	3.4 ± 1.3^{a}	4.0 ± 1.9^{a}
B (BA)	2.7 ± 1.4^{b}	2.3 ± 1.6^{b}
C (Kinetin)	1.5 ± 0.7^{c}	1.9 ± 1.2^{c}
D/control (no cytokinin)	1.4 ± 0.8^{c}	1.8 ± 1.3^{c}

Significant differences are indicated by superscripts with different letters ($p \leq 0.01$). Values are mean ± SD. n = 15

Table 4
ANOVA Summary Table for Shoot Number per Explant Data

Source of Variation	Sum of squares	df	Variance	F
(Between samples)	(11.927)	(7)		
Subcultures	0.002	1	0.002	0.028
Media (Cytokinins)	11.725	3	3.908	64.645**
Interaction	0.201	3	0.067	1.106
Within samples	6.771	112	0.060	
** indicates significance at P = 0.01				

Discussion

Cytokinins are growth regulators that are critical to the stimulation of shoot induction. Kinetin, 2iP and BA have been previously noted as some of the cytokinins that produced significant shooting response (Goda et al. 2023). Therefore, cytokinins are generally used on micropropagation media to promote *in vitro* shoot multiplication (Enkhbileg et al. 2019). 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 ± 11.4 mm and 10.5 ± 3.8 mm). Similar results were reported in *Grewia tenax* (Forssk.) Fiori (Daffalla et al. 2019) and another member of the Apocynaceae family, *Decalepis hamiltonii* Wight & Arn. (Giridhar et al. 2005) in which 2iP induced the greatest shoot proliferation of all three cytokinins. Kozak et al. (2021) 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 (Dey et al. 2020) and *Decalepis arayalpathra* (Ahmad et al. 2018) BA generated the best results for optimal shoot proliferation. According to Enkhbileg et al. (2019), the difference in results could be due to the effects of cytokinins differing depending on the plant species.

Conclusions

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 proliferation. Therefore, of the three cytokinins investigated 2iP produced the best results at stimulating shoot multiplication and growth of *Orbea semota*.

Abbreviations

2iP: 6-γ, γ-Dimethylallylamino purine

ANOVA: Analysis of variance

BA: 6-Benzylaminopurine

MS: Murashige and Skoog

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was financially supported by the Scientific Research Council.

Authors' contributions

KR 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). CS participated in the analysis and interpretation of the data and provided general supervision of the research. MW shared in the research proposal and design of the study. RF shared in the research proposal and provided general supervision of the research. All authors read and approved the final manuscript.

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.

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Figures

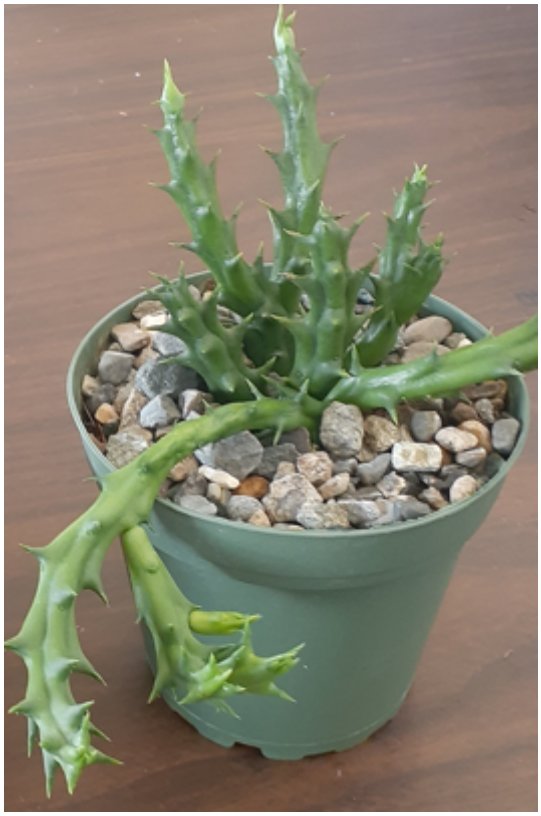


Figure 1

Branched stems of *Orbea semota*.

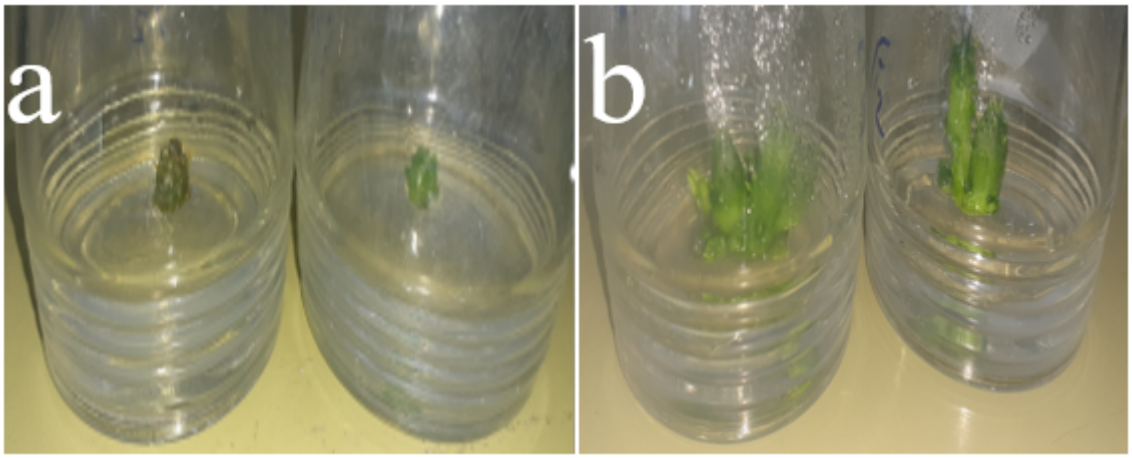


Figure 2

(a) Initiation of young shoots of 3-6mm in length on initiation media (b) Shoot proliferation on multiplication media (Medium A) after 8 weeks in culture.

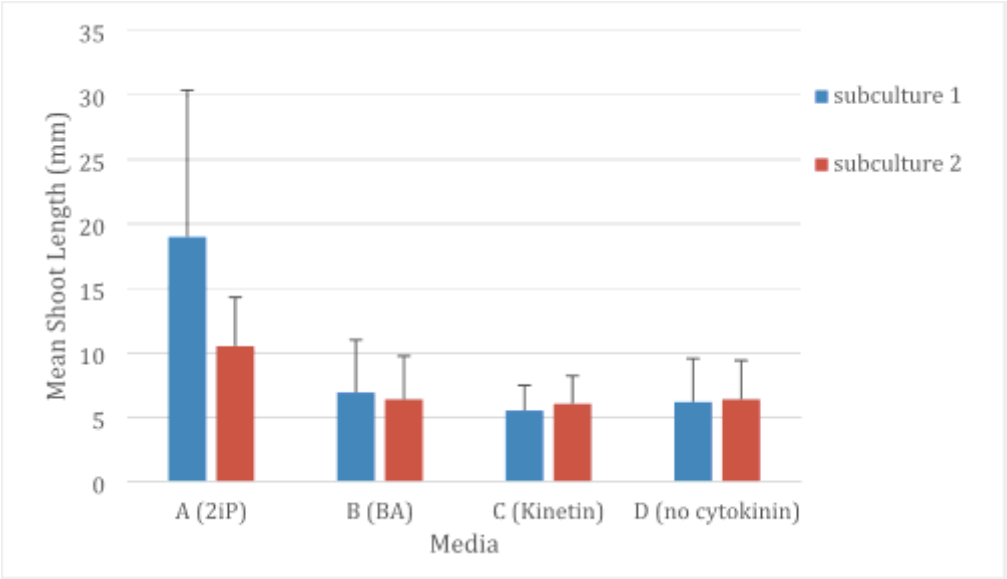


Figure 3

Differences in shoot length among hormone-supplemented media.

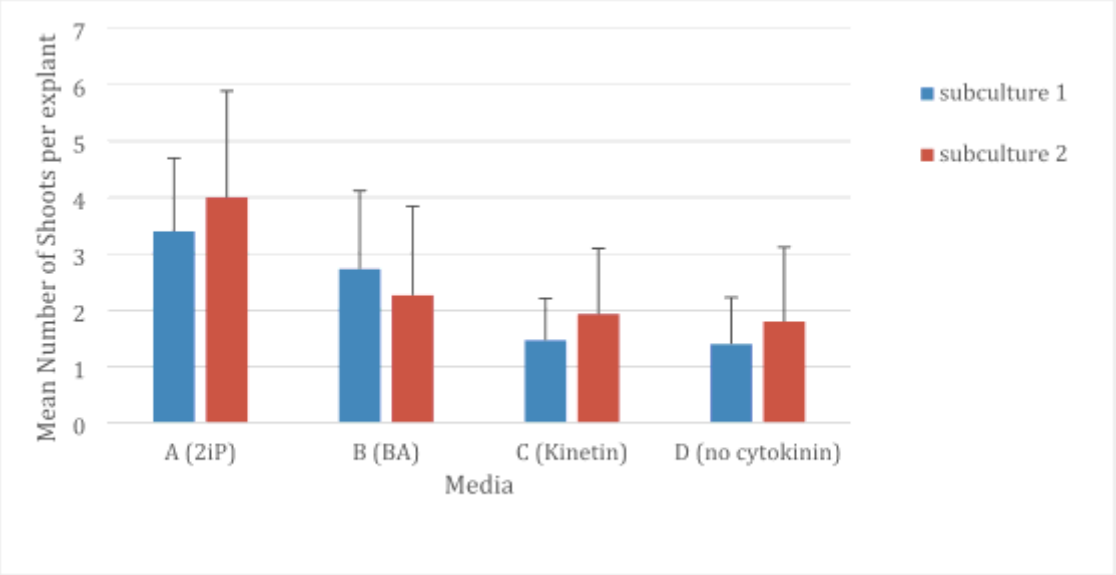


Figure 4

Differences in shoot number per explant among hormone-supplemented media.