Proceedings of Science and Mathematics
Faculty of Science, Universiti Teknologi Malaysia

Vol. 6, 2022, Page 45-50

Shoot Regeneration and Antioxidant Activity of *Orthosiphon Stamineus* fromNodal Segments

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Abstract
Orthosiphon stamineus (*O. stamineus*), often known as 'misai kucing', is an antioxidant-rich plant. Field-grown *O. stamineus* is limited to meet the high demand since people consume it as an herbal tea. *In vitro* culture is an alternative to yield high shoot biomass with good antioxidant activity of this species. Therefore, a study was carried out to see how plant growth regulators (PGR) affected the frequency of shoot regeneration, shoot biomass, and antioxidant activity of *O. stamineus* cultivated *in vitro*. On MS plates supplemented with 1–4 mg L^{-1} BAP, nodal explants (1 cm long) were grown. For 6 weeks, all cultures were incubated and subcultured every two weeks. FRAP and DPPH tests were used to measure antioxidant activity. The nodal explants treated with 4 mg L^{-1} BAP produced the most shoots (15.80 ± 0.76) and shoot length (6.63 ± 2.32 cm), according to the results. As for antioxidant activities, the highest FRAP value (7200.00 ± 103.02 µM Fe (II)) and lower EC_{50} (56.65 ± 0.17 µg mL^{-1}) were obtained for 4 mg L^{-1} BAP as compared to other treatments. In conclusion, PGR did enhance shoot regeneration of *in vitro O. stamineus* and the antioxidant activities.
Keywords: *Orthosiphon stamineus* shoot; regeneration; plant growth regulators, FRAP and DPPH.

Introduction

Orthosiphon stamineus is a species of *Orthosiphon* that can be found in China and Eastern Tropical Asia. In Malaysia, *O. stamineus* is called as 'misai kucing'. The tea extract can be used to treat kidney stones a urinary tract infections (UTI) that aren't too serious [1]. This species also has several pharmacological activities such as antioxidant [2], and antiproliferative [3]. These may be due to antioxidant substances present such as diterpenes, flavonoids, and phenolic acids [4]. Due to the values this species possessed, there has been a demand to yield it largely in a short period of time.

Stem cuttings are typically used to produce biomass of field-derived *O. stamineus.* However, according to Yaacob et al. [5], traditional propagation would not be able to meet considerable market demand. In this study, shoot regeneration through plant tissue culture and 6-Benzylaminopurine (BAP); a plant growth regulator (PGR) are two alternate strategies for increasing both *O. stamineus* biomass production and its antioxidant activity. Shoot regeneration is the ability to regenerate an entire shoot system from a tissue fragment or a single cell. It can be enhanced by the use of PGR; which is normally is a plant hormone that targets proliferation of certain explant such as stem, leaf, or root.

A study made by Muhammad Aeiman Farhan Arziallah was observed to affect both biomass and antioxidant content in shoot regeneration of *Persicaria odorata* when 2 mg L^{-1} of BAP + 0.2 mg L^{-1} naphthalene acetic acid (NAA) was added [6]. The effect of BAP and the antioxidant activity on *O. stamineus* was examined in this study.

Materials and methods

Plants of *O. stamineus* were cultivated and cared for in the greenhouse at UTM's Faculty of Science. The initial components were a six-week-old *in vitro* plant. *O. stamineus* nodal segments were surface sterilized. Field-grown *O. stamineus* nodal segments were removed and rinsed for 30 minutes under running tap water before being immersed in 70% (v/v) ethanol for 30 seconds and agitated for 45 minutes in a combination of 30% (v/v) Clorox + Tween 20. *In vitro* plants were kept in a tissue culture environment at 25°C for a 16-hour photoperiod with a light intensity of 1000 lux. Following that, the explants were rinsed six times with sterilized distilled water [5]. For treatment with BAP, six-week-old *in vitro* plants' nodal segments (1 cm) were placed on Murashige and Skoog (MS) plates treated with various BAP concentrations (1.0, 2.0, 3.0, and 4.0 mg L^{-1}). Each plate had five explants. There were five repetitions of each treatment. All cultures were cultured at the same temperature. After six weeks of cultivation, number of shoots produced per explant, percentage of shoot regeneration, length of shoots and weight of shoots were obtained.

After biomass was harvested, the biomass had to be extracted. To eliminate dust, plant samples were washed numerous times with deionized water and dried in a 60°C oven. Every hour, dried samples were weighed until a steady weight was attained. The samples were ground into a powder and then blended in a 1:50 (w/v) ratio with deionized water. To remove the debris, the mixture was put into a Falcon tube and centrifuged at 6000 rpm for 20 minutes. Filter paper was used to filter the mixture (Whatman 1). The filtrate was placed into a Falcon tube, which was freeze-dried for 24 hours in a freeze drier. The filtrate was then diluted in deionized water to 2 mg mL^{-1} and used for further analysis [4].

In this study, the antioxidant analyses consisted of two assays, 2,2-diphenyl-1-picryl-hydrazyl-hydrate, DPPH, and Ferric Reducing Antioxidant Power, FRAP. The DPPH assay was carried out according to Ojha et al. [1]. A 96-well microplate was filled with 100 µL of ethanol absolute. Before serial dilution from column 2 to column 10, 100 µL of the 2mg mL^{-1} sample was added to column 2. Every time 100 µL was pipetted from one column to the next, it was repeated until the last 100 µL was pipetted out. From column 2 to column 12, 100 µL of 0.04 % DPPH was added to rows A-E. At 517 nm, the absorbance was measured. Then, FRAP assay was carried out according to Ojha et al. [1]. Adding 900 µL of deionized water to 100 µL of the sample resulted in a dilution of 2 mg mL^{-1}. Before adding 200 µL of FRAP reagent to each column of a 96-well microplate, 100 µL of the diluted sample was placed in each column. At 593 nm, the absorbance was measured.

IBM SPSS Statistics 20 was used to statistically evaluate the data. The EC_{50} values for the DPPH assay were calculated using one-way ANOVA with Dunnett and Tukey HSD as post-hoc testing. Dunnett's test was used to compare each of the treated samples to L-ascorbic acid, which served as a single control, with the other groups' results expected to be lower than the control value [7].

Results and discussion

Effect of PGR on *O. stamineus* nodal segment shoot regeneration percentage

Figure 1 demonstrates *O. stamineus* shoot regrowth from nodal segments treated with 1-4 mg L^{-1} BAP. *O. stamineus* six-week-olds were given 1 – 4 mg L^{-1} BAP. The *in vitro* leaves and stem had a tiny, smooth, and green appearance. Shoot morphology changed from green to yellowish green as BAP content increased from 1 to 4 mg L^{-1}. The bushiness of the shoots decreased. True leaves were formed by nodal segments treated with 1 and 2 mg L^{-1} BAP, and leaf diameters were larger than those produced by 3 and 4 mg L^{-1} BAP. The size of the leaves became smaller and tapered when treated with 3 mg L^{-1} and 4 mg L^{-1}. Small and tapered shoots could be due to vitrification which was a result of increasing cytokinin concentrations.

The control culture had the deepest green shoots, as seen in Figure 1 (a). When compared to the treated culture, the shoots grew loosely to each other. Shoots cultured at 1 mg L^{-1} BAP, on the other hand, were bushier than the control. The serrate leaf margin in 1 mg L^{-1} BAP appeared to be more significant than the even margin in the control culture. The culture generated in the presence of 2 mg L^{-1} BAP contained both light green and yellowish-green leaves. The green colour became lighter as the BAP concentration increased.

The leaf had a needle-like morphology and grew very near to each other at 3 and 4 mg L^{-1} BAP. The leaves got thinner and less bushy as the concentration of BAP increased. The shoots got more transparent, shorter, and brittle as time went on. When a plant culture's water uptake is extremely high, this is known as hyperhydricity. Fresh weight inconsistency was caused by hyperhydricity in the shoot culture.

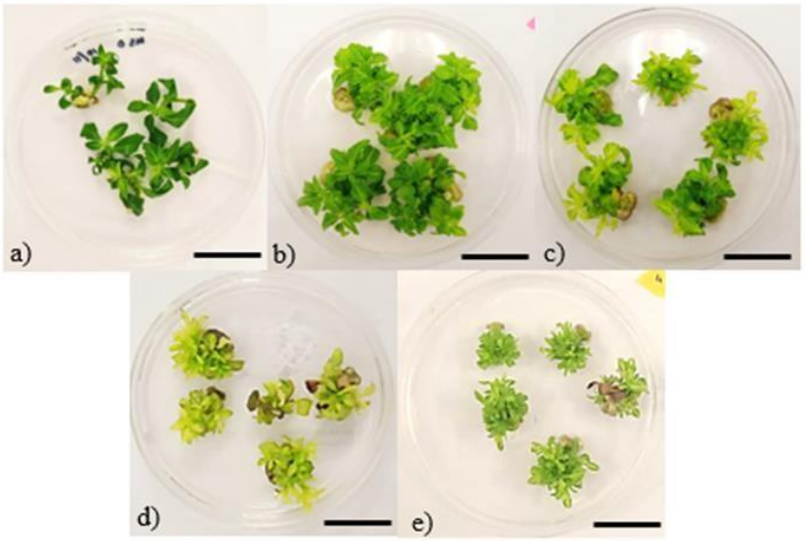

Figure 1 Shoots regenerated from nodal segments of *O. stamineus* a) no PGR, b) 1 mg L^{-1} BAP, c) 2 mg L^{-1} BAP, d) 3 mg L^{-1} BAP, e) 4 mg L^{-1} BAP. Scale: 1cm = 2.25 cm.

Based on the number of shoots, percentage of shoot regeneration (percent), shoot length (cm), and fresh weight, Table 1 indicates the influence of BAP (1-4 mg L^{-1}) on shoot regeneration (g).

Table 1: Effect of BAP on shoot induction of *O. stamineus.*

BAP Concentration (mg L^{-1})	No. of shoots produced per explant	Percentage of shoot regeneration (%)	Shoot length (cm)	Shoot Fresh weight (g)
0 (Control)	2.24 ± 0.19^{a}	100^{a}	2.70 ± 0.35^{a}	0.17 ± 0.02^{a}
1	6.40 ± 0.07^{b}	100^{a}	6.70 ± 0.09^{b}	0.41 ± 0.03^{c}
2	10.2 ± 1.73^{b}	100^{a}	5.40 ± 0.38^{b}	0.38 ± 0.03^{c}
3	$13.32 \pm 1.04^{b,c}$	100^{a}	5.51 ± 0.39^{b}	0.64 ± 0.20^{d}
4	$15.80 \pm 0.76^{b,c}$	100^{a}	$6.63 \pm 2.32^{b,c}$	0.31 ± 0.02^{c}

Data were expressed as mean ± standard error mean (SEM) of analysis (N=15). Different letters (a,b,c,d) show significant level between means at *p<0.05. The same letter indicates no significant difference between the means (p>0.05). The data analyzed is from one-ANOVA sample test; Post-Hoc test (Bonferroni).

BAP concentrations increased, resulting in an increase in the number of shoots. BAP is a cytokinin that has been utilized to stimulate and propagate shoots from nodal segments, as well as to increase the biomass of *O. stamineus* shoots. When treated with 4 mg L^{-1} of BAP, the largest number of shoots (15.80 ± 0.76) was achieved, compared to the control (2.24 ± 0.19). According to Kousalya and Narmatha Bai [8], the best concentration of BAP for *Canscora decussata* was 2 mg L^{-1}, with a total number of shoots of 30.20 ± 6.53. When BAP concentration was increased to 3 mg L^{-1}, however, the number of shoots drastically decreased to 19.80 ± 6.45. BAP at 1 mg L^{-1} resulted in longer shoots (6.70 ± 0.09 cm) compared to the control (2.70 ± 0.35 cm). Above 1 mg L^{-1} BAP, however, there was no significant change in shoot length. The length and quantity of shoots were not proportionate to the increase in cytokinin concentrations, according to an experiment done by Ashraf et al. [9]. The optimum for shoot fresh weight was attained at 3 mg L^{-1} BAP (0.64 ± 0.20 g), while the control generated the lowest.

Antioxidant assay

DPPH assay

The ability of antioxidant molecules to quench DPPH molecules, which act as free radicals and change the colour of DPPH from deep purple to yellow, is measured in the DPPH test (DPPH-H). Both field-grown and *in vitro* culture extracts were tested for antioxidant activity. With ascorbic acid as the standard compound, the EC_{50} values of field-grown and BAP treated *O. stamineus* are shown in Figure 2.

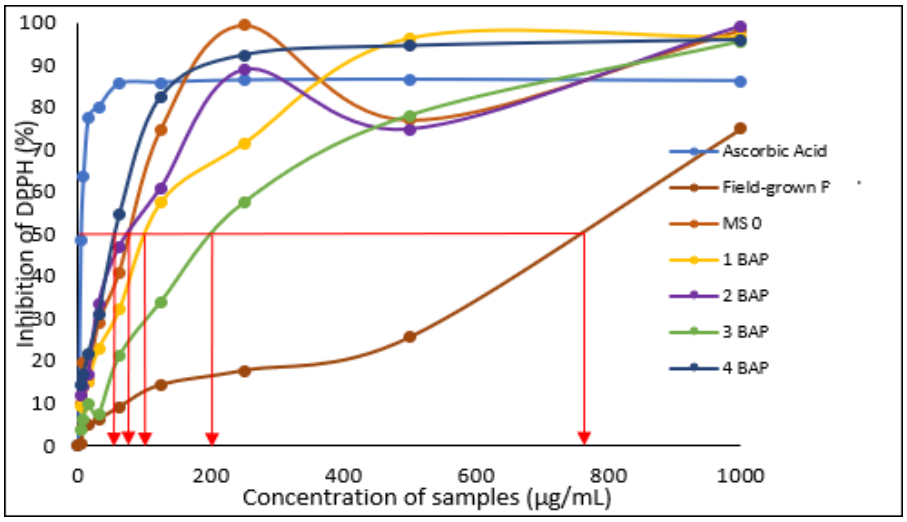

Figure 2 A comparison of inhibition of DPPH (%) between field-grown and *in vitro* culture of *O. stamineus* (1 – 4 mg L^{-1} BAP). (Red arrow represents EC_{50} values for all samples).

Red arrow in Figure 2 shows DPPH values at 50%. EC_{50} is an effective concentration of any sample for 50% inhibition of DPPH by hydrogen ions from antioxidant compounds. Based on Figure 2, at 100% inhibition of DPPH, *in vitro* shoot culture had higher antioxidant activity than field-grown plants. According to Kedare and Singh [10], antioxidant compounds were soluble in water and fat. In addition, insoluble ones are bound at the cell wall of the plant extract. Hence, they may not be free to react with DPPH and completion of the reaction will not reach in a reasonable time. Therefore, instead of waiting for 100% reaction, the activity of 50% scavenging activity is chosen for measuring the antioxidant potential.

At 500 g mL^{-1}, both 2 BAP and the control showed a decrease in DPPH inhibition (percent). However, as the reaction reached 100%, the activity climbed to 1000 g mL^{-1}. The antioxidant properties of phenolic compounds are due to their side chain functional groups. Because of the range of interactions between the side chain and the OH phenolic group, different conformations of an antioxidant compound have variable stability to scavenge DPPH molecules [11]. This could explain why MS 0 and 2 mg L^{-1} BAP therapies differ.

The low value of EC_{50} indicates high antioxidant activity. Overall, the EC_{50} of BAP-treated samples was lower than that of the field-grown sample. The EC_{50} value for L-ascorbic acid, a standard chemical, was the lowest. At a 50% reaction, increasing BAP treated culture did not improve antioxidant activity at the same time. Furthermore, ascorbic acid is an excellent reducer and is engaged in both metabolic and repair processes. This could imply that plant tissue culture had a significant impact on the antioxidant capacity of the plant extract. If the concentration was increased over 4 mg L^{-1} BAP, however, there might be an ideal EC_{50} value.

FRAP assay

Another test to measure the antioxidant capability of *O. stamineus* shoot biomass cultivated in the field and *in vitro* is the FRAP assay. The Fe^{3+} - TPTZ combination is reduced to Fe^{2+} - TPTZ complex in this test, rendering the colourless solution blue. Table 2 shows the FRAP values of purple-flowered *O. stamineus* plants grown in the field and BAP-treated samples.

Table 2: FRAP values for field-grown and *in vitro* culture of BAP treated of purple variety of *O. stamineus.*

Sample	FRAP µM Fe (II)/g
Field-grown extract	1733.33 ± 101.98^{a}
MS 0	3019.05 ± 109.83^{b}
1 mg L^{-1} BAP	4085.71 ± 75.59^{c}
2 mg L^{-1} BAP	4742.86 ± 103.02^{d}
3 mg L^{-1} BAP	$4447.62 \pm 98.80^{c,d}$
4 mg L^{-1} BAP	7200.00 ± 103.02^{e}

One-ANOVA sample test; Post-Hoc test (Bonferroni) in comparison of FRAP against samples of field-grown of purple variety of *O. stamineus* and *in vitro O. stamineus* (MS0, 1 – 4 mg L^{-1} BAP). Data were expressed as mean ± standard error mean (SEM) of analysis (N=15). Different letters (a,b,c,d) shown significant level between means at *p<0.05. The same letter indicates no significant difference between the means (p>0.05).

One-ANOVA sample test; Post-Hoc test (Bonferroni) in comparison of FRAP against samples of field-grown of purple variety of *O. stamineus* and *in vitro O. stamineus* (MS0, 1 – 4 mg L^{-1} BAP). Data were expressed as mean ± standard error mean (SEM) of analysis (N=15). Different letters (a,b,c,d) shown significant level between means at *p<0.05. The same letter indicates no significant difference between the means (p>0.05).

The antioxidant potentials of shoot biomass from nodal segments of *O. stamineus* were positively influenced using elicitors, such as BAP. The FRAP values of the *in vitro* shoot biomass were higher than those of the field-grown sample. The FRAP values increased considerably when the explants were treated with 4 mg L^{-1} BAP. The blue coloured complex became more vivid as the FRAP values increased, indicating that a larger concentration of antioxidants led Fe^{3+} to be reduced to Fe^{2+}.

Conclusion

In conclusion, this study shows that from 1 – 4 mg L^{-1} BAP, 4 mg L^{-1} produced the most shoots and the lowest fresh weight value (g). Every BAP concentration resulted in a 100% regeneration of the shoot. 4 mg L^{-1} BAP shoot culture had the lowest EC_{50} value in the DPPH assay. The antioxidant activity of the BAP-treated shoot culture was also higher than that of the field-cultivated culture. Furthermore, the antioxidant activity of 4 mg L^{-1} BAP was the greatest in the FRAP testing. Industrially, 4 mg L^{-1} could be used to yield *O. stamineus* biomass in a large amount with high antioxidant activity.

Acknowledgement

The authors would like to thank Universiti Teknologi Malaysia (UTM Transdiciplinary Research Grant (UTM-TDR); Vot No. 06G70) for financial support and Plant Biotechnology Laboratory, Faculty of Science for laboratory facilities.

References

[1] Ojha, S., Raj, A., Roy, A. and Roy, S. 2018. Extraction of total phenolics, flavonoids and tannins from *Paederia foetida* l. Leaves and their relation with antioxidant activity. Pharmacognosy Journa*.* 10(3): 541–547.

[2] Selamoglu, Z., Amin, K., Ugur, S. and Ozgen, O. 2018. Plant Secondary Metabolites with Antioxidant Properties and Human Health.

[3] Dolečková, I., Rárová, L., Grúz, J., Vondrusová, M., Strnad, M. and Kryštof, V. 2012. Antiproliferative and antiangiogenic effects of flavone eupatorin, an active constituent of chloroform extract of *Orthosiphon stamineus* leaves. Fitoterapia. 83(6): 1000–1007.

[4] Abdullah, M. Z., Mohd Ali, J., Abolmaesoomi, M., Abdul-Rahman, P. S. and Hashim, O. H. 2017. Anti-proliferative, *in vitro* antioxidant, and cellular antioxidant activities of the leaf extracts from *Polygonum minus* Huds: Effects of solvent polarity. International Journal of Food Properties. 20(1):846–862.

[5] Yaacob, J. S., Othman, R., Ali, H. and Karsani, S. A. (2018). Production of coloured callus in *Orthosiphon stamineus* Benth and antioxidant properties of the extracted pigment. Pigment and Resin Technology. 47(3): 196–207.

[6] Muhammad Aeiman Farhan Arziallah. 2021. Shoot regeneration and antioxidant activity of *Persicaria odorata* for silver nanoparticles synthesis. Master thesis. Universiti Teknologi Malaysia (UTM), Malaysia.

[7] Aritonang, H. F., Koleangan, H. and Wuntu, A. D. (2019). Synthesis of silver nanoparticles using aqueous extract of medicinal plants, (impatiens balsamina and lantana camara) fresh leaves and analysis of antimicrobial activity.International Journal of Microbiology. 2019 (8642303).

[8] Kousalya, L. and Narmatha Bai, V. (2016). Effect of growth regulators on rapid micropropagation and antioxidant activity of *Canscora decussata* (Roxb.). Asian Pacific Journal of Reproduction. 5(2): 161–170.

[9] Ashraf, M. F., Aziz, M. A., Kemat, N. and Ismail, I. 2014. Effect of cytokinin types, concentrations and their interactions on *in vitro* shoot regeneration of chlorophytum borivilianum sant. & fernandez. Electronic Journal of Biotechnology. 17(6): 275–279.

[10] Kedare, S. B. and Singh, R. P. 2011. Genesis and development of DPPH method of antioxidant assay. Journal of Food Science and Technology. 48(4): 412–422.

[11] Ichikawa, K., Sasada, R., Chiba, K. and Gotoh, H. 2019. Effect of side chain functional groups on the DPPH radical scavenging activity of Bisabolane-type phenols. Antioxidants. 8(3).