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Elicitation of *Orthosiphon stamineus* cell suspension culture for enhancement of phenolic compounds biosynthesis and antioxidant activity

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ABSTRACT

Our previous studies had showed that there was variation in the total phenolic contents with anti-oxidant activities of the field grown plants of *Orthosiphon stamineus* Benth (Lamiaceae). This study was hence carried out to evaluate the phenolic compound biosynthesis and the anti-oxidant activities of elicitor treated cells (ec) of *O. stamineus* compared to the mature plants (mp), *in vitro* plantlets (tcp) and the non-treated cells (nc). The total phenolic content was determined with Folin–Ciocalteu reagent, while the anti-oxidant activity was evaluated with DPPH and TEAC methods. The anti-oxidant activities of crude extracts decreased in the order of ec > tcp > nc > mp. Among the five tested elicitors, chitosan (150 mg/l) supplemented into the cell proliferation medium, MS + 1.0 mg/l NAA + 1.0 mg/l 2,4-D, enhanced the anti-oxidant activity and increased the total phenolic content of *O. stamineus* cell cultures. The anti-oxidant activity was correlated positively with the total phenolic content of the chitosan elicited cultured cells. This study indicated that cell suspension cultures of *O. stamineus* could be used as an alternative system for sustainable and consistent production of biochemical with anti-oxidant activity.

1. Introduction

Many plants have anti-oxidant and free radical scavenging capabilities. Chemical compounds in plants that contribute to anti-oxidant and free radical scavenging activities include phenolics, terpenoids, nitrogenous compounds and vitamins (Cai et al., 2004). Studies showed that many natural anti-oxidants possess anti-inflammatory, anti-atherosclerosis, anti-tumour, anti-mutagenic, anti-carcinogenic, anti-bacterial and anti-viral properties (Halliwell, 1994; Halliwell et al., 1995; Yang et al., 2001; Sun et al., 2002).

Orthosiphon stamineus Benth. (Lamiaceae), commonly known as Misai Kucing or Cat's Whiskers in Malaysia and Singapore, has been used as a traditional medicine for many centuries in Southeast Asia. It is used as a medicinal herb for disorders such as nephritis, nephrolithiasis, hydronephrosis, hypertension, vesical calculi, arteriosclerosis, gout, rheumatism, diabetes, oliguria and inflammation (Perry, 1980; Wiart, 2002; Olah et al., 2003). The leaves were found to possess diuretic (Beaux et al., 1999; Olah et al., 2003) and anti-hypertensive activities (Kazuyoshi et al., 2000). They also can prevent oxidative inactivation of 15-lipoxygenase (Lyckander and Malterud, 1996).

Bioactive polyphenols in plants have attracted interest due to their ability to protect the human body from the oxidative stress associated with many diseases such as cancer, cardiovascular diseases and ageing (Robards et al., 1999). *O. stamineus* contains several active compounds, such as terpenoids and polyphenols, that contribute to its therapeutic effects. Lipophilic flavonoids isolated from this plant have radical-scavenging activity towards the diphenylpicrylhydrazyl radical and inhibit activity of 15-lipoxygenase (Schut and Zwaving, 1993). The flavones, sinensetin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone isolated from *O. stamineus* showed diuretic activity in rats (Arafat et al., 2008). Diterpenes isolated from plants in the *Orthosiphon* genus have anti-inflammatory properties, prevent proliferation of rat thoracic aorta cells and inhibit nitric oxide effect (Masuda et al., 1992; Tezuka et al., 2001; Awale et al., 2002a,b, 2004). Rosmarinic acid is an anti-oxidant found in plants of the Lamiaceae family, including *O. stamineus*. Treatment with rosmarinic acid can reduce anxiety or depression produced by stress (Takeda et al., 2002). Due to its numerous uses, many herbal products containing *O. stamineus* dried plant material are currently produced and sold in Malaysian markets.

O. stamineus is conventionally propagated through cuttings and seeds. Studies had showed that field-grown *O. stamineus* plants

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produced inconsistent bioactive compounds (Lee, 2003). This problem, together with the variation of plant growth in the fields, has resulted in insufficient uniform plant material to meet the market demand. *In vitro* culture techniques such as plant cell suspension cultures can be used as an alternative means to produce secondary metabolites (Kutney, 1996; Luthfi et al., 2004; Chan, 2005; Koay et al., 2005). Two cell lines of *O. stamineus* were successfully selected and identified to produce high amounts of rosmarinic acid (Lim et al., 2007). However, the phenolic compounds, with anti-oxidant and free radical scavenging activities, produced by cell cultures of *O. stamineus* have not been evaluated.

In general, many plant cell cultures often do not produce sufficient amounts of the required bioactive compounds. In many cases, the production of secondary metabolites from plant cell cultures can be enhanced with the use of biotic and abiotic elicitors. Often complex biological preparations such as yeast extract, microbial cell wall preparations, polysaccharides, proteins and fatty acids are used as biotic elicitors. Abiotic elicitors are predominantly inorganic salts and physical factors such as ultraviolet light, detergents and metal ions (Thielfall and Whitehead, 1988; Wu et al., 2001). Both these biotic and abiotic elicitors induce product accumulation not only in intact plants but also plant cell cultures as a result of their defensive and protective reactions. Though elicitation has proven to enhance the production of secondary metabolites in plant cell cultures but the exact mechanism of elicitation is not fully understood (Namdeo, 2007).

Hence, the objectives of this study were to evaluate (i) the total phenolic content, (ii) free radical scavenging activity, and (iii) total anti-oxidant potential of the *in vitro* cell cultures of *O. stamineus* treated with different elicitors and (iv) to compare them with mother plants and *in vitro* plantlets.

2. Materials and methods

2.1. Plant materials

Three *O. stamineus* plant materials were prepared – field-grown mature plants, *in vitro* plantlets and cell suspension cultures.

2.1.1. Field-grown plants

Five-months-old mature plants of *O. stamineus* were collected from the nursery of the School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. A herbarium specimen of a mature plant was prepared and voucher specimen number 10106 was assigned and deposited at the Herbarium of the School of Biological Sciences, Universiti Sains, Malaysia. The collected plant materials were from three batches and were air-dried at room temperature (24–26 °C) until the weight was constant.

2.1.2. In vitro plantlets

Nodal segments of the field-grown *O. stamineus* were used as explants to establish the *in vitro* plantlets. Aseptic nodal segments were obtained following surface-sterilization procedure of Lee and Chan (2004a). The *in vitro* plantlets were propagated by culturing the aseptic nodal segments on MS (Murashige and Skoog, 1962) gelled medium supplemented with 2 mg/l benzyladenine (BA) and 7.5 g/l agar (Algas, Chile). The pH of the medium was adjusted to 5.7–5.8 prior to autoclaving at 121 °C and 1.06 kg/cm^2 for 13 min. All cultures were maintained at 25 ± 2 °C in a culture room with continuous light from cool white fluorescent lamps at 35 μmol/m^2/s. After five weeks, the plantlets from three batches of cultures were harvested and dried at room temperature (24–26 °C) for 12–14 days until constant weight was attained.

2.1.3. Cell suspension culture

Cell suspension cultures of *O. stamineus* were prepared from leaf-derived friable callus. The leaves of 5-week-old *in vitro* plantlets were used to induce friable callus according to Lee and Chan (2004b). The cells were cultured in 100 ml Erlenmeyer flasks containing 30 ml liquid MS medium supplemented with 1.0 mg/l 1-napthalene acetic acid (NAA) and 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Cultures were placed on a rotary shaker (G10 Gyrotary Shaker®, New Brunswick Scientific, USA) set at 120 rpm in a culture room maintained at 25 ± 2 °C under continuous light with a light intensity of 35 μmol/m^2/s. Three set of cell cultures were prepared. The cells were harvested after 12 days of culture by vacuum filtration through Whatman® No. 1 filter paper. The collected cells were dried at room temperature (24–26 °C) 12–14 days until constant weight was attained.

2.2. Elicitation of cell suspension culture

Casein hydrolysate (Duchefa, The Netherlands), sucrose, sodium chloride (NaCl), chitosan (Fluka, Germany) and yeast extract (Bacto™, France) were used to study the elicitation effect on the anti-oxidant activity and the production of phenolic compounds by the cell suspension cultures of *O. stamineus*. Casein hydrolysate, sucrose and NaCl were added to the cell proliferation medium (MS + 1.0 mg/l NAA + 1.0 mg/l 2,4-D) before autoclaving at 1.05 kg/cm^2 with 121 °C for 13 min. The final volume of the medium was 30 ml in 100 ml Erlenmeyer flasks with the concentration ranging from 0.3 to 3.0 g/l for casein hydrolysate (0.3, 0.6, 0.9, 1.2, 1.5 and 3.0 g/l), from 0 to 75 g/l for sucrose (0, 15, 30, 45, 60 and 75 g/l), and from 1.0 to 5.0 g/l for NaCl (1.0, 2.0, 3.0, 4.0 and 5.0 g/l). Chitosan and yeast extract were added aseptically to the cell cultures after 11 days of culture (24 h before the cells were harvested). Different concentrations of chitosan and yeast extract were prepared and autoclaved at 1.05 kg/cm^2 with 121 °C for 13 min before each of them was added into 30 ml of 11 days old cells of *O. stamineus* to make the final concentration of 25 mg/l, 50 mg/l, 75 mg/l, 100 mg/l and 150 mg/l of chitosan and 0.25, 0.5, 0.75, 1.0 and 1.5 g/l of yeast extract. As a control, 1.0 ml of sterile distilled water was added to the 11-day-old cell suspension culture. The experiment for each elicitation study was repeated three times. All cells that were treated with an elicitor were harvested by vacuum filtration after 12 days of culture. The harvested cells were air-dried at room temperature (24–26 °C) until constant weight was attained.

2.3. Preparation of extracts

The mature plant and the *in vitro* plantlet dried material from each batch were grounded with mortar and pestle into powder form. Crude extracts were prepared by soaking 1 g of the plant material (mature plant, *in vitro* plantlets and dried cells) in 20 ml of methanol for 24 h with agitation at 24 °C. The extracts were then centrifuged at 3000 rpm at 4 °C (Kubota micro refrigerated centrifuge, Japan). The supernatant was decanted and this supernatant was used to determine DPPH radical-scavenging activity, total anti-oxidant activity and total phenolic content.

2.4. Analytical methods

2.4.1. DPPH radical-scavenging assay

The DPPH radical-scavenging assay was used as modified in Braca et al. (2001), Kumaran and Karunakaran (2006) and Yam et al. (2006). A fresh crude extract of l00 μl was pipetted into 96 well plates and a serial dilution was prepared with distilled deionized water. Then 200 μl of 0.2 mM DPPH solution (in methanol) was pipetted into each well and the plate was incubated at room temperature (24–26 °C) for 30 min. Absorbance (OD517) of the mixture