UNIVERSITI PUTRA MALAYSIA

GROWTH, PHYTOCHEMICAL AND ANTIOXIDANT ACTIVITY OF Orthosiphon stamineus BENTH. IN RESPONSE TO ORGANIC AMENDMENT, FERTILIZER AND HARVEST DATE

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By

ESTHER YAP SHIAU PING

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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DEDICATION

Dedicated to my beloved parents, Yap Lian Huat and Teoh Sok Em, my sister, Estina Yap Shiau Yih for their endless love, support, understandings, sacrifices, motivation, advice and encouragement.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

GROWTH, PHYTOCHEMICAL AND ANTIOXIDANT ACTIVITY OF *Orthosiphon stamineus* BENTH. IN RESPONSE TO ORGANIC AMENDMENT, FERTILIZER AND HARVEST DATE

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Chairperson : Siti Hajar Ahmad, PhD
Faculty : Agriculture

Orthosiphon stamineus have been identified by Malaysian Department of Agriculture with the potential to be developed as complementary and alternative medicine. *O. stamineus* acts as a diuretic agent and has nephroprotective, antifungal, antimicrobial and antipyretic properties. It contained chemical markers, such as sinensetin (SEN) and rosmarinic acid (RA), with beneficial effects on consumer's health. Malaysian soils are predominantly kaolinitic clays and sandy due to weathering and is unsuitable for plant growth. Thus, it is important to incorporate organic soil amendments before planting to improve the soil. The objective of the first experiment was to determine the rates of soil amendments (rice husk biochar (BC): 0, 5 and 10 t/ha and chicken manure (CM): 0, 2.5 and 5 t/ha) and harvesting week (HW) that could produce the maximum yield and phytochemicals. The experiment was conducted as a potted experiment using a randomized complete block design in a three factorial arrangement of treatments with four replications and three subsamples each. There was a significant quadratic increase in plant height as harvesting time was increased, irrespective of soil amendments. Plant fresh weights were significantly affected by interactions between BC × CM × HW, whereby BC 0 t/ha and CM 5 t/ha at week 8 after transplanting gave the highest yield among all the treatments. There was a significant linear relationship between BC × HW for plant dry weight. The plant dry weight showed differences of 15%, 20% and 42% between week 6 and 8 at each BC rate. Both total phenolic and flavonoid contents were also affected by interactions between BC × CM × HW. 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity showed a significant quadratic response in the interaction between BC × HW whereby week 7 produced maximum DPPH activity. Sinensetin was significantly reduced by 63% at week 4 as compared to week 8, irrespective of soil amendments. The combination of BC 5 t/ha, CM 2.5 t/ha and HW 8 produced highest biomass yield, as HW 8 gave 36% higher yield than week 6, although SEN yield was not significantly different between week 6 and 8. The second experiment was carried out to determine the optimum fertilizer rate, FR (0, 100, 200, 300 and 400 kg/ha N) and fertilizer type, FT (plant- and animal-based) that could produce the maximum yield and phytochemicals. The selected treatment combination (BC 5 t/ha and CM 2.5 t/ha at HW 8) from the first experiment was used for soil

preparation. The potted experiment was conducted using a RCBD in a two factorial arrangement of treatments with four replications and three subsamples each. Both fresh and dry weights showed a significant quadratic trend against FR, irrespective of FT applied, with optimum rates at 323 kg/ha N and 219 kg/ha N, respectively. Total flavonoid contents (TFC) and DPPH activity were significantly affected by interaction between FR × FT. DPPH activity and TFC showed a quadratic trend for plant-based fertilizer with optimum rate at 218 kg/ha N and 244 kg/ha N, respectively. Chemical markers, RA and SEN, were also affected by interaction between FT × FR. Plant-based fertilizer showed a reduction in both the chemical markers as fertilizer rates increased. However, for animal-based fertilizer, the trend was quadratic, whereby the concentrations of RA and SEN were decreased by 61% and 13%, respectively, with increasing fertilizer rate from 0 kg/ha N to 200 kg/ha N. A gradual decrease, thereafter an increased in response to fertilizer rate was seen. The results showed plant-based fertilizers at 300 kg/ha N is the optimum organic fertilizer order to achieve maximum yield and phytochemicals. In conclusion, the optimum yield of *O. stamineus* can be obtained by using 5 t/ha rice husk biochar and 2.5 t/ha chicken manure soil amendments, plant-based organic fertilizer of 300 kg/ha N and harvested at week 8 after transplanting.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PERTUMBUHAN, KANDUNGAN FITOKIMIA DAN AKTIVITI ANTIOKSIDA BAGI *Orthosiphon stamineus* BENTH. SEBAGAI TINDAK BALAS TERHADAP PERAPI ORGANIK, BAJA DAN MASA PENUAIAN

Oleh

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Orthosiphon stamineus telah dikenal pasti oleh Jabatan Pertanian Malaysia sebagai tumbuhan yang berpotensi untuk dibangunkan sebagai perubatan komplementari dan alternatif. *O. stamineus* bertindak sebagai agen diuretik dan melindungi buah pinggang, anti-kulat, anti-mikrob dan antipiretik. Ia mengandungi penanda kimia seperti sinensetin (SEN) dan asid rosmarinik (RA) yang memberi kesan baik terhadap kesihatan penggunanya. Sebahagian besar tanah di Malaysia terdiri daripada tanah liat kaolin dan berpasir yang disebabkan oleh proses luluhawa dan tidak sesuai untuk pertumbuhan pokok. Oleh itu, menggunakan perapi tanah adalah penting sebelum penanaman untuk membaikpulih keadaan tanah. Objektif kajian pertama adalah untuk menentukan kadar perapi tanah (sekam padi biochar (BC): 0 , 5 dan 10 t/ha dan tahi ayam (CM): 0 , 2,5 dan 5 t/ha) dan minggu penuaian (HW) yang dapat mengeluarkan hasil tanaman dan kandungan fitokimia yang maksimum. Kajian ini dijalankan sebagai eksperimen pasu yang menggunakan reka bentuk blok rawak lengkap dalam tiga susunan faktorial rawatan, dengan empat ulangan dan tiga sub-sampel setiap ulangan. Terdapat peningkatan kuadratik yang ketara dalam ketinggian tumbuhan seiring dengan peningkatan masa penuaian, tanpa dipengaruhi oleh aplikasi perapi tanah. Berat basah tanaman dipengaruhi dengan ketara oleh interaksi diantara BC × CM × HW, di mana BC 0 t / ha dan CM 5 t/ha pada minggu ke-8 selepas anak pokok dialih, memberikan hasil yang tertinggi di antara semua rawatan. Terdapat hubungan linear yang ketara antara BC × HW untuk berat kering tanaman. Berat kering tanaman menunjukkan perbezaan pada kadar 15%, 20% dan 42% diantara minggu ke-6 dan 8 bagi setiap kadar BC. Kedua-dua jumlah kandungan fenolik dan flavonoid turut dipengaruhi oleh interaksi diantara BC × CM × HW. Aktiviti 2,2- difenil -1- pikrilhidrazil (DPPH) yang memerangkap radikal bebas menunjukkan tindak balas kuadratik yang ketara dalam interaksi antara BC × HW manakala minggu ke-7 menghasilkan aktiviti DPPH yang maksimum. Kandungan SEN telah berkurang dengan ketara sebanyak 63% pada minggu ke-4 berbanding minggu ke-8 , tanpa mengambil kira aplikasi perapi tanah. Gabungan BC 5 t/ha , CM 2.5 t/ha dan HW 8 menghasilkan hasil biojisim tertinggi , kerana HW 8 memberikan hasil 36 % lebih tinggi daripada minggu ke-6 , walaupun hasil SEN tidak jauh berbeza antara minggu ke-6 dan ke-8. Kajian kedua telah dijalankan untuk menentukan kadar optimum baja, FR (0 , 100, 200 , 300 dan 400 kg/ha N) dan jenis baja, FT (berasaskan tumbuhan dan berasaskan haiwan) yang boleh mengeluarkan hasil dan kandungan fitokimia yang maksimum. Gabungan rawatan yang dipilih (BC 5 t/ha , CM 2.5 t/ha dan HW 8) daripada percubaan pertama telah digunakan untuk penyediaan tanah. Kajian di dalam pasu telah dijalankan menggunakan RCBD dalam rawatan dua susunan faktorial, dengan empat ulangan dan tiga sub-sampel. Kedua-dua berat basah dan kering menunjukkan tren kuadratik yang ketara berbanding FR, tanpa mengira aplikasi FT, dengan kadar optimum pada 323 kg/ha N dan 219 kg/ha N masing-masing. Jumlah kandungan flavonoid (TFC) dan aktiviti DPPH dipengaruhi dengan ketara oleh interaksi antara FR × FT. Aktiviti DPPH dan TFC menunjukkan corak kuadratik untuk baja berasaskan tumbuhan dengan kadar optimum pada 218 kg/ha N dan 244 kg/ha N , masing-masing. Penanda kimia, RA dan SEN, turut dipengaruhi oleh interaksi antara FT × FR. Baja yang berasaskan tumbuhan menunjukkan pengurangan dalam kedua-dua penanda kimia seiring dengan peningkatan kadar baja. Walau bagaimanapun, baja berasaskan haiwan telah menunjukkan ciri kuadratik, di mana kepekatan RA dan SEN telah menurun dengan kadar baja yang semakin meningkat diikuti dengan penurunan secara beransur-ansur, selepas itu meningkat sebagai tindak balas kepada kadar baja. Hasil kajian menunjukkan baja berasaskan tumbuhan pada 300 kg/ha N adalah kadar baja organik yang optimum untuk mencapai hasil dan kandungan fitokimia yang maksimum. Kesimpulannya, hasil optima *O. stamineus* boleh didapati dengan menggunakan 5 t/ha sekam padi biochar, 2.5 t/ha tahi ayam, baja berasaskan tumbuhan pada 300 kg/ha N dan dituai pada minggu 8 selepas pemindahan anak pokok.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree Master of Science. The members of Supervisory Committee were as follows:

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TPC	Total phenolic contents
TFC	Total flavonoid contents
DPPH	2,2-diphenyl-1-picrylhydrazyl
w/w	Weight over weight
WHO	World Health Organization
RA	Rosmarinic acid
SEN	Sinensetin
BC	Rice husk biochar
t/ha	Tonnes per hectare
cm	Centimeter
kg	Kilogram
g	Gram
RCBD	Randomized complete block design
kg/ha	Kilogram per hectare
ANOVA	Analysis of variance
DMRT	Duncan multiple range test
LSD	Least significant differences
°C	Degree celsius
g/plant	Gram per plant
ml	Milliliter
FC	Folin-Ciocalteu
µl	Microliter
Na_2CO_3	Sodium carbonate
nm	Nanometer
GAE	Gallic acid equivalent
$NaNO_3$	Sodium nitrate
$AlCl_3$	Aluminium chloride
NaOH	Sodium hydroxide
QE	Quecertin equivalent
µm	Micrometer
mm	Milimeter
kg/plant	Kilogram per plant
cm/plant	Centimeter per plant
mg	Miligram
g	Gram
DW	Dry weight
N	Nitrogen
CM	Chicken manure
NKEA	National Key Economic Area
PMF	Polymethoxylated flavone
IFOAM	International Federation of Organic Agriculture Movement
DOA	Department of Agriculture
mg/kg	miligram per kilogram
ALP	Alkaline phosphatase
AST	Aspartate aminotransferase
g/kg	gram per kilogram
USDA	United States Department of Agriculture
DHPL	3,4-dihydroxyphenyllactic acid
CEC	Cation exchange capacity

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Orthosiphon stamineus has been identified as one of the high value product under the National Key Economic Area (NKEA) introduced by the Ministry of Agriculture and Agro-Based Industry (Anon, 2015). This is due to the high antioxidant compounds such as flavones, polyphenols, bioactive proteins and glycosidesin the plant which gives the plant its diuretic, hepatoprotective, antifungal, antimicrobial and antidiabetic roles in human health (Hossain et al., 2008; Adam et al., 2009; Alshawsh et al., 2011; Alshawsh et al., 2012; Elsnoussi et al., 2012). However, common agricultural practices were unable to produce optimum antioxidant compounds in regards to maximum yield. This is because the herb producers' focus is on the amount of yield that they can produce in order to meet the demands from the industry. However, the amount of yield produced does not quantify the amount of antioxidant compounds in the plant. Thus, it is important to find the most suitable agricultural practices that can provide optimum yield and also antioxidant properties.

The two main chemical markers which make *O. stamineus* a valuable medicinal herb are rosmarinic acid (RA) and sinensetin (SEN). Rosmarinic acid, a phenolic acid present in plants as a secondary metabolite, is a water-soluble ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, which can be found naturally in many plants, especially in the species of Boraginaceae and Lamiaceae (Petersen et al., 2003; Petersen, 2013). Rosmarinic acid has been reported to be an antioxidant, possess anti-inflammatory, antiapoptotic, antifibrotic, antimicrobial activity, neuroprotective and neurorescue effect (Jordan et al., 2012; Wang et al., 2012; Domitroric et al., 2013; Braidy et al., 2014). Sinensetin, a flavonoid is a rare polymethoxylated flavone (PMF) and is mostly found in citrus plants with 5-methoxy groups on the basic benzo-γ-pyrone skeleton with a carbonyl group at the C_4 position. Sinensetin has been reported to show anti-inflammatory activity, antioxidant potential and antiproliferative activity (Du and Chen, 2010; Jiang et al., 2012; Shin et al., 2012).

However, the amount of chemical compounds produced by herbs is irregular and insufficient due to poor soil condition and insufficient fertilization lack of water supply, irradiation and temperature (Hansen and Wold, 2010). Thus, it is often altered structurally to produce drugs that are potentially more active. Traditional medicine usages have been on the rise since the 1990s in many developed and developing countries (WHO, 2015). The reasons for this rise are due to lower costs of herbs as compared to synthetic drugs, the need for alternative treatments for drug-resistant pathogens and increasing popularity for products that are natural and environmental friendly (Bandaranayake, 2006). Thus, organic farming is the most likely answer to cultivating herbs for medicinal purposes. IFOAM (2008) defines organic agriculture as a production system that takes into consideration the health of soils, ecosystems and people, which discourage the use of inputs with adverse effects but relies on ecological processes, biodiversity and cycles adapted to local conditions. Chin et al. (2010)

reported that organic farming of tea plantation helps in increasing tea leaf growth by 23% and also major polyphenols by 38%. Organic inputs such as organic fertilizers and bio-organic fertilizers also showed an increase in fresh yield (20%), total phenolic content (45%) and total flavonoid content (51%) in sweet fennel (Salama et al., 2015). Organic fertilization also helps in the improvement of biomass yield and nutrient absorption in herbs at different levels of organic nitrogen fertilizers (Jennifer et al., 2015; Marcio et al., 2015).

The main step in organic farming is to ensure that the soil is healthy to be planted on. However, soils in Malaysia are lacking in organic matter and thus, soil amendments are needed to improve the soil characteristics. Soil amendments are defined as materials that are applied to the soil in order to correct major constraint other than low nutrient content in the soil (Anon, 2010).Organic amendments are used due to their organic matter content which acts as a carrier of utilizable energy and nutrients for the soil organisms. They also help to improve soil structure and porosity, increase water holding capacity of soils, improve aeration, reduce soil temperature fluctuations, storage of nutrients in exchangeable form and as provision of nutrients (Anon, 2010). Rice husk biochar and chicken manure are some examples of soil amendment, which can be used in agriculture. Both are used as a soil amendment because they are not only easily available and cheap, but also help to reduce heavy metals availability in the soil by increasing the soil pH (Masulili et al., 2010).

Besides organic farming, harvesting date or time is also crucial in ensuring the optimum biomass weight, phytochemical and antioxidant activities are achieved. Generally, crops that are for export purposes are usually harvested earlier as compared for consumption as fresh products or for local use, whereby quality attributes such as colour meets consumer preferences. Studies have been done on the impact of harvesting time on the phytochemical content of various crops. Lata et al. (2005) found that blueberries harvested in August had a lower content of phenolics (5%), flavonols (40%) and anthocyanins (2%) than those harvested in July. In another study in Hungary, the total phenolics in broccoli increased by 0.8% and antioxidant activity decreased by 21% when harvested during fall as compared to spring season (Pek et al., 2012). Lv et al. (2016) reported a decrease in oleanolic acid by 2% from the first to the third harvest in the year 2012 and the same trend can be seen in the year 2013 with a decrease of 13%. However, ursolic acid was inconsistent throughout the three harvests in the year 2012, but in the year 2013, a reduction of 9% was reported. These studies showed inconsistent results of phytochemicals and antioxidant activities in crops at different harvesting time. Harvesting time in most crops are planned in order to obtain the optimum levels of health-promoting compounds and thus, it is important to determine the suitable harvesting time in a particular crop.

Despite the high demand for quality and safe *O. stamineus* that are produced organically, not much research has been done to look at the effect of organic planting of *O. stamineus* on the yield, quality and safety. Therefore, the objectives for this study were to study the effects of (i) different types and rates of soil amendments for optimum yield, antioxidant compounds and activities, and chemical markers of *O. stamineus* and (ii) different types and rates of organic fertilizer for optimum yield, antioxidant compounds and activities, and chemical markers of *O. stamineus*. The hypothesis of the study are (i) combination of rice husk biochar and chicken manure soil amendment at 5 t/ha and 2.5 t/ha, respectively will provide the optimum yield. (ii) harvesting week 8 will produce the highest fresh and dry weights. (iii) plant-based organic fertilizer at 300 kg/ha N will give optimum yield.

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