UNIVERSITI PUTRA MALAYSIA

MAJOR PIGMENT AND FRAGRANT COMPOUNDS IN SELECTED ORCHIDS AND THE CORRELATION BETWEEN PHENYLALANINE AMMONIA LYASE (EC 4.3.1.5) ACTIVITY AND ANTHOCYANIN CONTENT

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By

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

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Orchidaceae family has become one of the important commercial commodities in agricultural industry worldwide, either as potted plants or as cut flowers due to the attractive colour produced in flower petals. However, the colour of the orchid flowers usually found to be non-patterned and non-uniformed between the species or in a single orchid plant. The major pigment synthesized in the orchid flower is commonly derived from anthocyanin family. Therefore, determining the distribution and characteristics of the key enzyme of phenylpropanoid pathway (phenylalanine ammonia lyase; PAL) in different orchid tissues and its correlation to anthocyanin content were the main objective in this study. Apart from that, the major fragrant compounds were also determined by using gas chromatography-mass spectrometry (GC-MS).

Results obtained from the studies revealed that anthocyanins content were found to be the highest when compare to β-carotenes and chlorophylls in coloured orchid petals (*Dendrobium* Sonia 17, *Vanda* Mimi Palmer, *Phalaenopsis bellina* and *Oncidium* Sharry Baby) at a range of 0.07 to 0.95 mg/g fresh weight. For non-anthocyanin containing orchids (*D.* Savin White, *P. bellina* var. alba and *V.* White (*V.* Velthuis x *Coeletis alba*)), the highest pigment content present in their petals was chlorophyll at a range of 0.05 to 0.34 mg/g fresh weight. Anthocyanin and β-carotene pigment were not detected in *in vitro* orchid seedlings, protocorm-like bodies (PLBs) and leaves. The major pigment found to be present in *in vitro* seedlings, PLBs and leaves of orchids was chlorophyll at a range 0.05 to 0.34 mg/g fresh weight. The total β-carotene content was determined to be highest in *O.* Taka petals (0.09 ± 0.01 mg/g fresh weight). Additionally, the major anthocyanidin pigment in orchid flowers was analyzed using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Malvidin (0.113 ± 0.00 mg/g fresh weight) and petunidin (0.117 ± 0.00 mg/g fresh weight) were present in *D.* Sonia 17 petals, while, petunidin (0.109 ± 0.00 mg/g fresh weight) and delphinidin (0.096 ± 0.00 mg/g fresh weight) were observed in *P. bellina*. Only delphinidin was detected in *V.* Mimi Palmer (0.129 ± 0.02 mg/g fresh weight) and *O.* Sharry Baby (0.08 ± 0.02 mg/g fresh wight) petals. Besides, the major fragrant compounds was also determined in fragrant orchids (*V.* Mimi Palmer, *P. bellina*, *P. bellina* var. alba and *O.* Sharry Baby).

A study was also carried out to determine the correlation of PAL activity and anthocyanin content in orchid flowers. PAL is the first enzyme involved in anthocyanin biosynthesis. Its activity was found to be positively correlated with the total anthocyanin content in the coloured orchid petals. The PAL activity and total anthocyanin content in the full bloomed of coloured orchid flowers was at a range 0.12 to 0.36 nmol/min/mg protein and 0.07 to 0.95 mg/g fresh weight, respectively. Apart from that, from the Pearson correlation analysis, PAL activity was found to be significant positive correlated with the anthocyanin content during the development of flower of *D*. Sonia 17, *V*. Mimi Palmer, *P. bellina* and *O*. Sharry Baby ($r=0.989$, $p<0.05$; $r=0.867$, $p<0.05$; $r=0.989$, $p<0.05$ and $r=0.966$, $p<0.05$, respectively). Therefore, higher PAL activity is associated with higher anthocyanin content in petals of coloured orchids.

In a subsequent study on characterization of PAL, it was observed that PAL from orchid flower, seedlings, PLBs and leaves showed similar optimum pH (pH 8.5), temperature (30 °C) and incubation time (15 min) among each other. However, PAL was found to have different V_{max} and K_m values towards L-phenylalanine as its substrate. The K_m and V_{max} values of PAL towards L-phenylalanine were found within a range of 100 to 264 µM and 0.32 to 0.98 µmol/min.mg protein, respectively. Besides, PAL was also activated by the addition of Mg^{2+}, Mn^{2+} and Ca^{2+} ions in its reaction mixture.

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

PIGMEN DAN SEBATIAN WANGI UTAMA DI DALAM ORKID-ORKID TERPILIH DAN HUBUNG KAIT ANTARA AKTIVITI FENILALANINA AMONIA LIASE (EC 4.3.1.5) DAN KANDUNGAN ANTOSIANIN

Oleh

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Keluarga orchidaceae telah menjadi salah satu barang dagangan komersil terpenting di dalam industri pertanian di seluruh dunia, samada sebagai tanaman berpasu atau sebagai bunga keratan kerana warna yang menarik yang dihasilkan di dalam kelopak bunga. Walaubagaimanapun, warna bunga orkid selalunya didapati tidak sama corak dan tidak seragam antara spesies atau di dalam satu pokok orkid. Pigmen utama disintesis di dalam bunga orkid pada umumnya berasal dari keluarga antosianin. Oleh itu, penentuan taburan dan ciri-ciri enzim utama bagi tapak jalan fenilpropanoid (fenilalanina amonia liase; PAL) di dalam tisu orkid berlainan dan hubungkaitnya dengan kandungan antosianin adalah merupakan objektif utama dalam kajian ini. Selain daripada itu, sebatian wangi utama juga ditentukan dengan menggunakan kromatografi gas-spektrometri jisim (GC-MS).

Keputusan yang diperolehi dalam kajian ini mendedahkan bahawa kandungan antosianin didapati tertinggi apabila dibandingkan dengan pigmen β-karotin dan klorofil di dalam kelopak bunga orkid berwarna (*Dendrobium* Sonia 17, *Vanda* Mimi Palmer, *Phalaenopsis bellina* dan *Oncidium* Sharry Baby) pada julat di antara 0.05 ke 0.07 mg/g berat basah. Pigmen antosianin dan β-karotin didapati tidak dikesan di dalam anak-anak pokok *in vitro*, jasad meyerupai protokom (PLBs) dan dedaun orkid. Pigmen utama terdapat di dalam anak-anak pokok *in vitro*, PLBs dan dedaun orkid adalah klorofil pada julat 0.05 ke 0.34 mg/g berat basah. Jumlah β-karotin dikenalpasti tertinggi dalam kelopak bunga *O*. Taka (0.09 ± 0.01 mg/g berat basah). Selain itu, pigmen antosianidin di dalam bunga orkid dianalisis dengan menggunakan kromatografi lapisan nipis (TLC) dan kromatografi cecair berprestasi tinggi (HPLC). Malvidin (0.113 ± 0.00 mg/g berat basah) dan petunidin (0.117 ± 0.00 mg/g berat basah) didapati di dalam kelopak bunga *D*. Sonia 17, manakala, petunidin (0.109 ± 0.00 mg/g berat basah) dan delphinidin (0.096 ± 0.00 mg/g berat basah) dikenalpasti di dalam kelopak bunga *P. bellina*. Hanya delphinidin dikesan di dalam kelopak bunga *V*. Mimi Palmer (0.129 ± 0.02 mg/g berat basah) dan *O*. Sharry Baby (0.08 ± 0.02 mg/g berat basah). Selain daripada itu, sebatian wangian utama juga dikesan di dalam orkid-orkid wangi (*V*. Mimi Palmer, *P. bellina*, *P. bellina* var. alba and *O*. Sharry Baby).

Kajian juga dijalankan untuk menentukan hubungkait antara aktiviti PAL dan kandungan antosianin di dalam bunga orkid. PAL adalah enzim pertama terlibat di dalam biosintesis antosianin. Aktivitinya didapati berkaitan secara positif dengan jumlah kandungan antosianin di dalam kelopak bunga orkid berwarna. Aktiviti PAL dan jumlah kandungan antosianin di dalam bunga orkid berwarna yang kembang penuh adalah masing-masing pada julat 0.12 ke 0.36 nmol/min/mg protein dan 0.07 ke 0.95 mg/g berat basah. Selain daripada itu, daripada analisis hubung kait Pearson, aktiviti PAL didapati berkaitan secara positif dengan kandungan antosianin semasa perkembangan bunga *D*. Sonia 17, *V*. Mimi Palmer, *P. bellina* dan *O*. Sharry Baby (r=0.989, p<0.05; r=0.867, p<0.05; r=0.989, p<0.05 and r=0.966, p<0.05, masing-masing). Oleh itu, tinggi aktiviti PAL adalah berkaitan dengan tinggi kandungan antosianin di dalam kelopak bunga orkid berwarna.

Dalam kajian yang seterusnya ke atas pencirian PAL, didapati bahawa PAL daripada bunga, anak-anak pokok, PLBs dan dedaun menunjukkan pH (pH 8.5), suhu (30 °C) dan masa pengeraman (15 min) optima yang sama antara satu sama lain. Walaubagaimanapun, PAL didapati mempunyai nilai V_{max} dan K_m yang berbeza terhadap L-fenilalanina sebagai substratnya. Nilai K_m dan V_{max} PAL terhadap L-fenilalanina masing-masing didapati pada julat 100 ke 264 µM dan 0.32 ke 0.98 µmol/min/mg protein. Selain itu, PAL juga diransang dengan penambahan ion-ion Mg^{2+}, Mn^{2+} dan Ca^{2+} ke dalam campuran tindak balas katalitiknya.

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I certify that a Thesis Examination Committee has met on 28 December 2009 to conduct the final examination of Azzreena binti Mohamad Azzeme on her thesis entitled "Major Pigment and Fragrant Compounds in Selected Orchids and the Correlation between Phenylalanine Ammonia Lyase (EC 4.3.1.5) Activity and Anthocyanin Content" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master Science.

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Date: 11 February 2010

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

AZZREENA BINTI MOHAMAD AZZEME

Date: 11 March 2010

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LIST OF ABBREVIATIONS

mg	Milligram
g	Gram
ml	Milliliter
L	Liter
mM	Millimolar
nmol	Nanomole
µmol	Micromole
%	Percentage
°C	Degree celsius
v/v	Volume per volume
min	Minutes
FW	Fresh weight
ABA	Abscisic acid
CHI	Chalcone isomerase
CHS	Chalcone synthase
DFR	Dihydroflavonol 4–reductase
DHK	Dihydrokaempferol
DHM	Dihydromyricetin
DHQ	Dihydroquercetin
EDTA	Ethylenediaminetetraacetic acid
Co-A	Co-enzyme A

HAL	Histidine ammonia lyase
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
MeOH	Methanol
PAL	Phenylalanine ammonia lyase
PLBs	Protocorm-like bodies
SAM	S-adenosyl-L-methionine
TLC	Thin layer chromatography
UFGT	UDP-glucose:flavanoid glycosyltransferase
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

Orchids belong to the largest family of flowering plants in the world. According to Croix (2008), more than 20 000 to 30 000 species representing 900 genera of orchids have been found and recorded. These species are classified as *Phalaenopsis*, *Vanda*, *Dendrobium*, *Oncidium*, *Cattleya*, *Epidendrum*, *Phaphiopedilum*, *Brassica*, *Laelia*, and *Miltonia* (Choi et. al., 2006 and Zotz et. al., 2006). However, yearly breeding programs, carried out all over the world, have produced thousands of new genus of orchid hybrids from wild genera (species), such as *Aeriditis* (*Aerides* x *Doritis*), *Angrangis* (*Aerangis* x *Angraecum*), *Aranda* (*Arachnis* x *Vanda*), *Doritaenopsis* (*Doritis* x *Phalaenopsis*) and *Epicattleya* (*Cattleya* x *Epidendrum*) (Cullina, 2004). It was reported that, 100 000 hybrids of orchids have been produced over the last 150 years (Royal Horticultural Society, 2008; Roberts and Dixon, 2008) and over 808 species covering 111 genera are known to be indigenous to Peninsular Malaysia (Gunman and Yusof, 2006). Each species of orchids represents a different floral morphology of colours, shapes and sizes. Interestingly, some of them are scented (Choi et. al., 2006 and Zotz et. al., 2006). The development of different kinds of new hybrids is reflected by the preference of the consumers, market targets and seasons. Orchid production has become the main business in agriculture industry and its monetary value has significantly increased yearly. There is a great potential for orchids in domestic and international market (Federal Agriculture Marketing Authority (FAMA), 2008).

There are several orchid species which have been widely commercialized such as *Vanda* (Tatsuzawa et. al., 2004), *Phalaenopsis* (Huang et. al., 2004; Kosir et. al., 2004; Li et. al., 2006), *Dendrobium* (Khentry et. al., 2006), *Oncidium* (Jheng et. al., 2006), *Mokara*, *Aranda* and *Renanthera* (http://www.orchidasia.com). They are usually sold as potted plants and cut flowers.

V. Mimi Palmer, one of the most desirable orchids, is a hybrid of *V*. Tan Chay Yan and *V*. *tessellate*, originating in tropical Asia. It is a highly fragrant orchid, hardy and free flowering all year round (Motes and Hoffman, 2004). The planting materials for the said hybrid are in great demand for commercial production as well as for domestic gardens and landscaping. The production of *Dendrobium* is also increasing yearly. The plants are sold as cut flowers and potted plants for interior landscaping of hotels and restaurants (Tee et. al., 2003; Khentry et. al., 2006). The main attraction of *Dendrobium* is the flower spray, which has a wide range of colours, sizes and shapes, year-round availability and long flowering life of several weeks to months (Martin and Madassery, 2006). *Oncidium*, is a tropical orchid, which is native to Southeast Asia and Pacific area. Similarly, *Oncidium* comprised one of the top traded group of orchids which is being sold as potted plants and cut flowers (Chen and Chang, 2000; Jheng et. al., 2006). The atypical combination of their colour, yellow and accentuated with red made the *Oncidium* differ from the other orchid genera. The colour combination of the *Oncidium* flowers makes the genera have been chosen for chemical and molecular genetic analysis particularly in comparison with the other orchids (Hieber et. al., 2006). There is a hybrid in this family, which produced luscious chocolate or vanilla scent, *O*. Sharry Baby (*O*.

Jamie Sutton x *O*. Honolulu). This orchid is one of the most popular orchids in demand in the world (Fitch, 2004; Frowine, 2005).

There are many problems associated with the orchid industry. Firstly, the commercial market demands for cut-flower plants which produce long inflorescences with lots of large flowers as compared to the ones with short inflorescences and a few small flowers to fulfill the consumers' needs. Secondly, growers need uniformity in producing the product. This is generally due to the fact that most of the orchid plants have different growing habits and are blooming at different times. Finally, the market also prefers the flowers to be uniform in colours and are not patterned (Griesbach, 2002).

Variation and not patterned flower colour is due to the genes and the enzymes controlling the production of pigments in the plants. Thus, the functions of the related genes and enzymes need to be well characterized (Weisshaar and Jenkins, 1998). There are three major pigments, which are responsible for pigmentation in plants; chlorophyll is generally responsible for the colour green, while anthocyanins give red and blue, and carotenoids are responsible for yellow (Lewis et. al., 1998). Beautiful and attractive colours of flowers are mostly due to anthocyanins, which are produced in the petals (Yoshida et. al., 2003; Yoshikazu and Ohmiya, 2008). Anthocyanin is synthesized through phenylpropanoid pathway (Nielson et. al, 2002; Koes et. al., 2005). Phenylalanine ammonia lyase (PAL) catalyzes the first committed reaction for this pathway, which generate hundreds of different phenylpropanoid metabolites including anthocyanins, the main pigments in flowers. The relationship between PAL and anthocyanin synthesis in the plants are not well understood. PAL has been reported to have a positive correlation with the anthocyanin synthesis in grapes, strawberries and apples (Wang et. al., 2000). However, the role of PAL in regulating anthocyanins in apples remains controversial (Ju et. al., 1995; Ju et. al., 1997). The enzyme has multiple roles in plant metabolism such as for cell wall strengthening and repair (Imura et. al., 2001), flavonoids accumulation for colour and UV protection (Oyanagi and Ozeki, 2001; Skolaut and Retey, 2001), phytoalexin production for antimicrobial activity (Gomez-Vasquez et. al., 2004) and salicylic acid synthesis for signaling compounds (D'Cunha, 2005; Wen et. al., 2005; Chen et. al., 2006). Previous reports have revealed that, PAL enzyme was encoded by four to five isoform of genes (PAL 1, PAL 2, PAL 3 and PAL 4) (Cochrane et. al., 2004) which is believed to produce different isoforms of enzyme that is responsible for either anthocyanins or other phenolic compounds (Olsen et. al., 2008). Besides, there is a need to determine the distribution of this enzyme whether there is a link with anthocyanin content primarily in the flower petals. The result may contribute to the knowledge and establishment of this enzyme as a potential biomarker for flower colour in orchids. Apart from this, a preliminary analysis of fragrant compounds in fragrant orchids is carried out which may later help in formulating strategies to develop fragrant orchids.

The objectives of this study were:

1. To determine the major pigments and fragrant compounds present in selected orchids.
2. To determine the distribution and to characterize crude extract of PAL in selected orchids.
3. To establish a correlation between PAL activity and anthocyanin content in orchid petals.