

**TISSUE CULTURE, ULTRASTRUCTURAL STUDIES AND  
ESSENTIAL OIL ANALYSIS OF *Ocimum basilicum* L.**

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**FACULTY OF SCIENCE  
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AND ESSENTIAL OIL ANALYSIS OF *Ocimum basilicum* L.**

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## ABSTRACT

This study describes efficient protocols for coloured callus formation, *in vitro* shoot multiplication, complete plant regeneration, and *in vitro* flowering from leaf and shoot tip explants of *Ocimum basilicum* 'Sweet Thai', chemotype methyl chavicol. In addition, it reveals information on pollen and seed germinability, non-glandular and glandular trichome ultrastructures, and essential oil content and composition of the plant at different plant developmental stages. Leaf and shoot tip explants from two-month-old aseptic seedlings were induced to form coloured callus, multiple shoots, complete plants, and *in vitro* flowers on Murashige and Skoog (MS) medium supplemented with different plant growth regulators (PGRs) [6-benzyl-aminopurine (BAP) and gibberellic acid ( $GA_3$ ) either alone or in combination with  $\alpha$ -naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D)]. The highest percentage of purple callus formation (100%) from both leaf and shoot tip explants was recorded on MS medium supplemented with 0.5 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> NAA and also on MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> NAA. Whilst, the highest percentage of light green callus formation (100%) from both leaf and shoot tip explants was recorded on MS medium supplemented with 0.5 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> 2,4-D and also on MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> 2,4-D. The highest number of multiple shoots (mean 5 shoots per explant) was obtained from shoot tip explants cultured on MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP. Multiple shoots showed 100% rooting on half-strength MS basal medium. Shoot tip explants exhibited the highest percentage of *in vitro* flowering (40%) on MS medium supplemented with 1.0 mg l<sup>-1</sup>  $GA_3$ . All (100%) of the multiple shoots flowered *in vitro* on MS medium supplemented with 1.0 mg l<sup>-1</sup>  $GA_3$ . The rooted plantlets were successfully acclimatized in vermiculite and established in a soil mixture (3 garden soil:

2 potting mix) in a field with an 80% survival rate, which further developed flowers *ex vitro* after 24 weeks. Relative to the mother plant, *in vitro* grown plants flowered at a younger stage of plant development but produced no seeds, and showed a lack of pollen germination, fewer fully-filled peltate glandular trichomes, lower essential oil content, and higher methyl chavicol content. *Ex vitro* grown plants flowered at an intermediate stage of plant development with seed formation, and showed nearly the same pollen and seed germinability, essential oil content, and methyl chavicol content as in the mother plant.

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## ABSTRAK

Kajian ini menerangkan protokol yang efisien bagi pembentukan kalus berwarna, penggandaan pucuk secara *in vitro*, regenerasi tumbuhan lengkap, dan pembungaan secara *in vitro* daripada eksplan daun dan hujung pucuk *Ocimum basilicum* 'Sweet Thai', kemotip metil chavikol. Di samping itu, ia juga mendedahkan maklumat tentang kebolehcambahan debunga dan biji benih, ultrastruktur trikoma tidak-berkelenjar dan berkelenjar, serta kandungan dan komposisi minyak pati dalam tumbuhan tersebut pada peringkat perkembangan tumbuhan yang berbeza. Eksplan daun dan hujung pucuk daripada anak benih aseptik berusia dua bulan diinduksi untuk membentuk kalus berwarna, pucuk berganda, tumbuhan lengkap, dan bunga *in vitro* dalam media Murashige dan Skoog (MS) yang ditambah dengan hormon tumbuhan yang berbeza [6-benzil-aminopurin (BAP) dan asid gibberellik ( $GA_3$ ) samada sendirian atau dalam kombinasi dengan asid  $\alpha$ -naftalenaasetik (NAA) atau asid 2,4-diklorofenoksiasetik (2,4-D)]. Peratusan pembentukan kalus ungu yang tertinggi (100%) daripada kedua-dua eksplan daun dan hujung pucuk dicatatkan dalam media MS yang ditambah dengan  $0.5 \text{ mg l}^{-1}$  BAP dan  $0.5 \text{ mg l}^{-1}$  NAA serta media MS yang ditambah dengan  $1.0 \text{ mg l}^{-1}$  BAP dan  $0.5 \text{ mg l}^{-1}$  NAA. Sementara itu, peratusan pembentukan kalus hijau terang yang tertinggi (100%) daripada kedua-dua eksplan daun dan hujung pucuk dicatatkan dalam media MS yang ditambah dengan  $0.5 \text{ mg l}^{-1}$  BAP dan  $1.0 \text{ mg l}^{-1}$  2,4-D serta media MS yang ditambah dengan  $1.0 \text{ mg l}^{-1}$  BAP and  $1.0 \text{ mg l}^{-1}$  2,4-D. Bilangan pucuk berganda yang tertinggi (min 5 pucuk per eksplan) diperoleh daripada eksplan hujung pucuk yang dikultur dalam media MS yang ditambah dengan  $1.0 \text{ mg l}^{-1}$  BAP. Pucuk berganda menunjukkan 100% pertumbuhan akar dalam media asas MS separuh-kekuatan. Eksplan hujung pucuk mempamerkan peratusan pembungaan secara *in vitro* yang tertinggi (40%) dalam media MS yang ditambah dengan  $1.0 \text{ mg l}^{-1}$   $GA_3$ . Kesemua

(100%) pucuk berganda berbunga secara *in vitro* dalam media MS yang ditambah dengan  $1.0 \text{ mg l}^{-1} \text{ GA}_3$ . Anak pokok yang berakar telah berjaya diaklimatisasi dalam vermikulit dan tumbuh dalam tanah campuran (3 tanah kebun: 2 campuran pot) di lapangan dengan 80% kadar keterusan hidup, dan seterusnya menghasilkan bunga secara *ex vitro* selepas 24 minggu. Berbanding dengan pokok induk, tumbuhan yang tumbuh secara *in vitro* berbunga pada peringkat perkembangan tumbuhan yang lebih muda tetapi tidak menghasilkan biji benih, serta menunjukkan tiada percambahan debunga, lebih sedikit trikom berkelenjar peltat yang terisi-penuh, lebih rendah kandungan minyak pati, dan lebih tinggi kandungan metil chavikol. Tumbuhan yang tumbuh secara *ex vitro* berbunga pada peringkat pertengahan perkembangan tumbuhan dan menghasilkan biji benih, serta menunjukkan kebolehcambahan debunga dan biji benih, kandungan minyak pati, dan kandungan metil chavikol yang hampir sama dengan pokok induk.

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*Aziemah Abdul Manan*



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## LIST OF SYMBOLS AND ABBREVIATIONS

|                                      |                                       |
|--------------------------------------|---------------------------------------|
| °C                                   | degree Celsius                        |
| °C min <sup>-1</sup>                 | degree Celsius per minute             |
| μl                                   | microlitre                            |
| μm                                   | micrometre                            |
| μmol m <sup>-2</sup> s <sup>-1</sup> | micromole per square metre per second |
| %                                    | percent                               |
| 2,4-D                                | 2,4-dichlorophenoxyacetic acid        |
| ANOVA                                | analysis of variance                  |
| BAP                                  | 6-benzyl-aminopurine                  |
| C                                    | carbon                                |
| cm                                   | centimetre                            |
| CO <sub>2</sub>                      | carbon dioxide                        |
| CPD                                  | critical point drying                 |
| CVOMT                                | chavicol O-methyltransferase          |
| FID                                  | flame ionization detector             |
| g                                    | gram                                  |
| GA                                   | gibberellin                           |
| GA <sub>3</sub>                      | gibberellic acid                      |
| GC-MS                                | gas chromatography-mass spectrometry  |
| HCl                                  | hydrochloric acid                     |
| l                                    | litre                                 |
| m                                    | metre                                 |
| mg l <sup>-1</sup>                   | milligram per litre                   |
| ml                                   | millilitre                            |

|                      |  |
|----------------------|--|
| ml min <sup>-1</sup> | millilitre per minute                  |
| mm                   | millimetre                             |
| mm <sup>2</sup>      | square millimetre                      |
| MS                   | Murashige and Skoog                    |
| N                    | normality                              |
| NAA                  | $\alpha$ -naphthaleneacetic acid       |
| NaOH                 | sodium hydroxide                       |
| OsO <sub>4</sub>     | osmium tetroxide                       |
| PGR                  | plant growth regulator                 |
| pH                   | power of hydrogen                      |
| psi                  | pounds per square inch                 |
| SE                   | standard error                         |
| SEM                  | scanning electron microscope           |
| SPL                  | SQUAMOSA PROMOTER BINDING-LIKE         |
| SPSS                 | Statistical Package for Social Science |
| W                    | watt                                   |

## CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

### 1.1 Introduction

*Ocimum basilicum* L. (sweet basil) belongs to the family Lamiaceae and is a multipurpose herb characterized by its rich and aromatic essential oil content. The plant genus (*Ocimum*) is primarily grown in tropical and subtropical regions in Asia, Africa, and Central and South America as a culinary herb and an attractive, fragrant ornamental (Carović-Stanko *et al.*, 2010). The aromatic leaves, flowers, and seeds of *O. basilicum* are added to foods and beverages for flavour, extracted as active ingredient for use in perfumes, soaps, cosmetics, and dental products, and included in traditional herbal medicines to treat fevers, headaches, kidney problems, gum ulcers, childbirths, rheumatoid arthritis, and menstrual irregularities (The Herb Society of America, 2003). Besides these traditional medical uses, recent scientific studies have demonstrated potent antioxidant (Jayasinghe *et al.*, 2003), antiviral (Chiang *et al.*, 2005), and anti-proliferative properties (Manosroi *et al.*, 2006) of compounds in *O. basilicum* essential oil and leaf extracts.

*O. basilicum* essential oil is a mixture of numerous compounds, mainly methyl chavicol (estragole), linalool, 1,8-cineole (eucalyptol), eugenol, and methyl cinnamate (Wesołowska *et al.*, 2012; Said-Al Ahl *et al.*, 2015). These compounds possess several biological activities. For example, methyl chavicol has antispasmodic (Coelho-de-Souza *et al.*, 1997), antimicrobial (Friedman *et al.*, 2002), and local anaesthetic properties (Silva-Alves *et al.*, 2013), linalool has anti-inflammatory properties (Peana *et al.*, 2002), 1,8-cineole has vasorelaxant properties (Lahlou *et al.*, 2002), and eugenol has antioxidant properties (Ogata *et al.*, 2000). However, the composition of *O. basilicum*

essential oil varies depending on the cultivar, and the taxonomy is complicated by the existence of chemotypes or chemical races that do not differ significantly in morphology (Carović-Stanko *et al.*, 2010). Lawrence (1988) classified four major chemotypes of *O. basilicum* based on essential oil composition: methyl chavicol-rich, linalool-rich, methyl eugenol-rich, and methyl cinnamate-rich. The developmental stage of the plant also influences the yield and composition of *O. basilicum* essential oil (Deschamps and Simon, 2010; Verma *et al.*, 2012).

Micropropagation provides an effective means of rapid propagation and large-scale production of uniform plants of selected cultivars while maintaining their genotype (Arikat *et al.*, 2004). The micropropagated plants may serve as efficient plant models to study the accumulation of volatile compounds at different plant developmental stages. Besides its potential to regenerate into a whole plant, the callus produced by *in vitro* culture can carry a special role for producing valuable medicinal and bioactive phytochemicals in large-scale from plants (Sen *et al.*, 2014). *In vitro* flowering can be a useful tool to study the flowering process, to accelerate breeding programs, and to optimize the commercial production of specific compounds from floral organs (Zeng *et al.*, 2013). The application of tissue culture for the production of secondary metabolites is beneficial, as this method enables rapid production of secondary metabolites due to rapid growth of cultures *in vitro*, year-round production of secondary metabolites irrespective of seasonal and climatic conditions, and avoidance of collecting endangered wild species (Arikat *et al.*, 2004).

Several authors have reported on the callus initiation, shoot multiplication, and *in vitro* flowering of *O. basilicum* (Phippen and Simon, 2000; Begum *et al.*, 2002; Sudhakaran and Sivasankari, 2002). However, the plant growth regulator (PGR)

requirements of this species for coloured callus initiation, shoot multiplication, and *in vitro* flowering vary in different cultivars and chemotypes. Studies about pollen and seed quality derived from the flowers of *in vitro* regenerated *O. basilicum* have not yet been documented. Structural and chemical information regarding non-glandular and glandular trichome ultrastructures and essential oil content and composition, respectively from *in vitro* regenerated *O. basilicum* are also very limited.

Therefore, the goal of this study was to identify the PGR or combination of PGRs that would best support induction of coloured callus formation, shoot multiplication, and *in vitro* flowering from leaf and shoot tip explants of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol, and to assess pollen and seed germinability, non-glandular and glandular trichome ultrastructure, and essential oil content and composition at different developmental stages. This information may facilitate mass production of coloured callus, plants, and flowers containing high levels of uniformity in the aspects of pollen and seed germinability, non-glandular and glandular trichome ultrastructure, and essential oil content and composition.



## 1.2 Literature Review

### 1.2.1 Description of *Ocimum basilicum* L.

*Ocimum basilicum* L. or commonly known as ‘sweet basil’ is a member of the genus *Ocimum*, which comprises more than 30 species of herbs and shrubs native to the tropical and subtropical regions of Asia, Africa, and Central and South America (Carović-Stanko *et al.*, 2010). The plant is also called as ‘selasih’ in Malay, ‘basilico’ in Italian, ‘raihan’ in Arabic, ‘babui tulsi’ in Hindi, and ‘luo le’ in Chinese. The taxonomic hierarchy for classification of *Ocimum basilicum* L. is as listed below:

|           |   |
|-----------|---|
| Kingdom:  | Plantae                                       |
| Division: | Magnoliophyta                                 |
| Class:    | Magnoliopsida                                 |
| Order:    | Lamiales                                      |
| Family:   | Lamiaceae                                     |
| Genus:    | <i>Ocimum</i> L.                              |
| Species:  | <i>Ocimum basilicum</i> L. (USDA, NRCS, 2015) |

*O. basilicum* is an annual or short lived perennial herb of 20-60 cm tall. It is described as aromatic in all aerial parts with stems erect or ascending, branching, square, woody at base; leaf blade narrowly ovate to elliptic, glandular-punctate, glabrous or with small hairs on veins beneath; flowers hermaphrodite, arranged in six-flowered verticils, in a lax inflorescence; calyx horizontal, downward-pointing, shortly tubular, bilabiate (two-lipped), upper lip entire, lower lip subequally four-lobed; corolla purple, pink, white or creamy yellow, funnel-shaped, bilabiate, upper lip subequally four-lobed, lower lip entire; stamens four; style bifid (split in two); and seeds black, ovoid, mucilaginous when wet (Paton, 1992).

### 1.2.2 Varieties of *Ocimum basilicum* L.

There is a wide diversity of *O. basilicum* cultivars (short form for “cultivated varieties”) around the world. The cultivars usually are produced by selection of plants for desirable characteristics and maintained through cultivation. Darrah (1980) classified *O. basilicum* cultivars in seven types: (1) tall slender types, which include the sweet basil group, (2) large-leafed, robust types, including ‘Lettuce Leaf’ also called ‘Italian’ basil, (3) dwarf types, which are short and small leafed such as ‘Bush’ basil, (4) compact types, also described ‘Thai’ basil, (5) *purpurascens*, the purple-coloured basil types with traditional sweet basil flavour, (6) purple types, such as ‘Dark Opal’ which has lobed-leaves with a sweet basil plus clove-like aroma, and (7) *citriodorum* types, which includes lemon-flavoured basils.

*O. basilicum* cultivars are of different distinct aromas. The distinctive scents and flavours of the many *O. basilicum* cultivars are related to the composition of essential oils found in the leaves and other parts of the plant (The Herb Society of America, 2003). These essential oil composition differences have created a chemotype or chemovar (short form for “chemical variety”) classification of *O. basilicum* species. A chemotype designation usually occurs when the plant is grown in a different area of the world or a distinct climate that alters the chemistry of the plants. Different chemotypes have been identified from accessions of *O. basilicum* based on major compounds of the essential oils. On the basis of more than 200 analyses of essential oils isolated from *O. basilicum*, Lawrence (1988) classified four major essential oil chemotypes of basil: (1) methyl chavicol-rich, (2) linalool-rich, (3) methyl eugenol-rich, and (4) methyl cinnamate-rich, and also numerous subtypes. Grayer *et al.* (1996) recognized five basic essential oil profiles in the *O. basilicum* plants investigated: (1) linalool as major compound, (2) methyl chavicol as major compound, (3) linalool and methyl chavicol as

major compounds, (4) linalool and eugenol as major compounds, and (5) methyl chavicol and methyl eugenol as major compounds.

The chemotypes of *O. basilicum* essential oil are commonly known by names based on geographical origins such as European, Egyptian, Reunion, Bulgarian, and Java basil oils (Simon *et al.*, 1999). The European type of basil oil contains linalool and methyl chavicol as the major constituents; Egyptian basil oil contains a higher concentration of methyl chavicol than linalool; Reunion basil oil from Comoro Islands, Madagascar, Thailand, and Vietnam contains a very high concentration of methyl chavicol; basil oil from Bulgaria, India, Guatemala, and Pakistan is rich in methyl cinnamate; and basil oil from Java, Russia, and North Africa is rich in eugenol (Simon *et al.*, 1999).

### **1.2.3 Uses of *Ocimum basilicum* L.**

*O. basilicum* has been well-recognized for its multipurpose use especially in the ancient Greek royalty, hence it is referred to as the ‘royal herb’ (Herbst, 2001). The aromatic leaves are used fresh or dried in various cuisines including pesto, stews, soups, rice, pasta, and salads, and the seeds are used in beverages (The Herb Society of America, 2003). *O. basilicum* is also a nice ornamental plant, valued for its attractive colours and flowers, and impressive scent. The essential oil of *O. basilicum* is used in aromatherapy and in foods, perfumes, soaps, cosmetics, and dental products (The Herb Society of America, 2003).

*O. basilicum* has been used in traditional medicine in countries around the world. In the Philippines, the leaves are poulticed onto fungal infections; in Salvador, *O. basilicum* is placed in the ears to cure deafness; and in Malaya, the leaf decoction may

be administered after childbirth, and the juice may be taken if the menses are delayed (Duke, 2001). In China, *O. basilicum* is used in traditional medicine for kidney problems, gum ulcers, and as hemostyptic in childbirth; and in India, it is used in treatments for earache, rheumatoid arthritis, anorexia, skin conditions, menstrual irregularities, and malaria (The Herb Society of America, 2003). Reported to have antispasmodic, galactogenic, and emmenagogue properties, Pamplona-Roger (2002) recommends that drinking leaf and flower infuse and essence can calm nervous digestive disorders, such as digestive spasms (stomach nervousness), aerophagia (excess of gas and belches), and nervous dyspepsia (slow digestion caused by nervous tension), increase the production of milk in breast-feeding women, and ease menstruation and the pain caused by uterine spasms or congestion.

The application of *O. basilicum* in medicine is not limited to traditional use only. *O. basilicum* has been studied scientifically under *in vitro* condition and the leaf extract was found to possess antioxidant activity due to high prevalence of phenolic compounds (Jayasinghe *et al.*, 2003). Extracts and selected pure constituents of *O. basilicum* exhibited a broad spectrum of antiviral activity, especially against coxsackievirus B1 (CVB1) and enterovirus 71 (EV71) (Chiang *et al.*, 2005). Aqueous extract of *O. basilicum* showed potent inhibitory activity against human immunodeficiency virus-1 (HIV-1) reverse transcriptase (Yamasaki *et al.*, 1998). Manosroi *et al.* (2006) reported anti-proliferative activity of *O. basilicum* essential oil in murine leukaemia (P388) cell line. Experiment carried out using mice has shown that the leaves of *O. basilicum* increased glutathione-S-transferase activity, enzyme for detoxifying chemical carcinogens in the stomach, liver, and oesophagus (Aruna and Sivaramakrishnan, 1990).

Essential oil of *O. basilicum* can also be a potential natural element to improve quality in agriculture and food industries. The essential oil has antifungal properties that inhibited mycelial growth of fungi, including *Aspergillus flavus* and *Aspergillus parasiticus*, with a lower toxic dose compared to some commercial fungicides and fumigants, and it was unaffected by temperature treatment, storage and increased inoculum (Dube *et al.*, 1989). It also proved to repel the agricultural pests *Allacophora foveicollis*. *O. basilicum* essential oil, with antimicrobial activity against microorganisms as *Aeromonas hydrophila* and *Pseudomonas fluorescens* could play a role in offering a natural alternative for the washing of selected fresh salad for enhanced preservation to replace or reduce the concentration of chlorine which commonly used to reduce the microbial load on the salad products (Wan *et al.*, 1998).

Three cultivars of *Ocimum basilicum*: Dark Opal, Sweet Thai, and Genovese have been found to be rich in anthocyanins (Nguyen *et al.*, 2010). Anthocyanins are water-soluble pigments that responsible for the colouration of flowers, fruits, and other plant parts, ranging from violet and blue to red (*The American heritage dictionary of the English language*, 2011). The pigment has potential not only as a natural colorant, but also as an antioxidant, to enhance visual acuity, to inhibit cancer and tumour formation, and to prevent obesity, diabetes, cardiovascular, and neurodegenerative disorders (Lila, 2004). The identification of anthocyanins in *O. basilicum* may bring to the manipulation of this species for high-amount production of anthocyanins from plants.

#### **1.2.4 Chemistry and biological activities of compounds in *Ocimum basilicum* L.**

Generally, *O. basilicum* contains linalool, methyl chavicol (estragole), 1,8-cineole (eucalyptol), eugenol, methyl cinnamate, geraniol,  $\beta$ -cadinene, cadinol, and  $\alpha$ -bergamotene as the main components in the essential oil (Wesołowska *et al.*, 2012;

Nurzyńska-Wierdak *et al.*, 2013, Said-Al Ahl *et al.*, 2015). However, the levels of these compounds vary depending on the cultivars and chemotypes. The compounds are reported to have several important biological activities. For example, methyl chavicol has antispasmodic properties that relaxes intestinal smooth muscle (Coelho-de-Souza *et al.*, 1997), bactericidal properties against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enterica* (Friedman *et al.*, 2002), and local anesthetic properties that blocks neuronal excitability by direct inhibition of voltage-gated activation of Na<sup>+</sup> channel conductance (Silva-Alves *et al.*, 2013). Linalool has anti-inflammatory properties that result in reduction of carrageenin-induced edema in rats (Pena *et al.*, 2002). Other compounds like 1,8-cineole and eugenol has vasorelaxant properties (Lahlou *et al.*, 2002) and antioxidant properties (Ogata *et al.*, 2000), respectively.

Seeds of *O. basilicum* liberate mucilage when steeped in water. The mucilage (9.3%) yields, on hydrolysis, uronic acid, glucose, xylose and rhamnose (Panda, 2010). The seeds also contain oil with the following fatty acid composition: unsaturated fatty acids including alpha-linolenic (43.8% to 64.8%), linoleic (17.8% to 31.3%), and oleic (8.5% to 13.3%); and saturated fatty acids including palmitic (6.1% to 11.0%) and stearic (2.0% to 4.0%) (Angers *et al.*, 1996).

*O. basilicum* essence however, if internally applied in high dose, can cause narcotic effects (Pamplona-Roger, 2002). Estragole (methyl chavicol) contained in *O. basilicum* oil was reported to be possibly carcinogenic; therefore, it is not recommended for pregnant women and children (The Herb Society of America, 2003).

### 1.2.5 Principles in plant tissue culture

Plant tissue culture, also referred to as *in vitro*, axenic, or sterile culture, is a technique used for the aseptic culture of cells, tissues, organs, and their components under defined physical and chemical conditions *in vitro* (Thorpe, 2013). The theoretical basis for plant tissue culture was proposed by Gottlieb Haberlandt in 1902 when he attempted to culture isolated mesophyll parenchyma cells of *Lamium purpureum*, but none of the cells were observed to divide. Nevertheless, he concluded that "...in this way, one could successfully cultivate artificial embryos from vegetative cells", thus established the totipotentiality of plant cells (Sussex, 2008). Haberlandt's idea and novel cell culture methods has pioneered advances in cell culture technology, and in 1965, Vasil and Hildebrandt achieved regeneration of whole plants from isolated single cells of tobacco (Bhojwani and Dantu, 2013).

Two concepts are central to understanding plant tissue culture: plasticity and totipotency. Plasticity is the ability of plants to alter their metabolism; growth and development in order to best suit their environment (Slater, 2008). Plant cells and tissues cultured *in vitro* generally exhibit a very high degree of plasticity, which allows one type of tissue or organ to be regenerated from another type. Totipotency is the ability of plant cells, given the correct stimuli, to express the total genetic potential of the parent plant (Slater, 2008). In other word, the concept of totipotency holds that all living cells have the capacity of regenerating a whole organism. In plant tissue culture, plasticity and totipotency enable plant cells to initiate cell division from almost any tissue of the plant and to regenerate lost organs or undergo different developmental pathways in response to particular stimuli. In this way, whole plants can be subsequently regenerated.

Plant regeneration from differentiated cells is generally preceded by the mature cells reverting back to meristematic state, a phenomenon termed ‘de-differentiation’, followed by divisions to form an unorganized callus (Bhojwani and Dantu, 2013). The callusing phase may be very brief or long, depending on the system. Regeneration of plants from callus or, sometimes, directly from the pre-existing meristematic cells is termed ‘re-differentiation’ (Bhojwani and Dantu, 2013). A mechanism of converting one type of differentiated cells directly to another type of differentiated cells without undergoing through a pluripotent state or progenitor cell type is referred to as ‘transdifferentiation’ (Xu *et al.*, 2014).

In broad terms, two methods of plant regeneration are widely used in plant tissue culture study: somatic embryogenesis and organogenesis. Somatic embryogenesis is the formation of embryos, which can develop into whole plants, from somatic plant cells (García-González *et al.*, 2010). These somatic embryos are formed either directly or indirectly. In direct somatic embryogenesis, the embryo is formed directly from the explant; while in indirect somatic embryogenesis, the embryo is formed via the intervening callus produced from the explant. The embryos may pass through four stages in their development: (1) globular stage, (2) heart stage, (3) torpedo stage, and (4) cotyledonary stage. Organogenesis is the formation of plant organs from explants or callus in order to form whole plants (García-González *et al.*, 2010). Organogenesis may be direct, if the organogenic shoot is directly obtained from the explant, or indirect, if the organogenic process occurs from previously formed callus in the explant.

Plant tissue culture is associated with a wide range of applications. These applications can be divided into nine broad areas, namely: (1) study of cell behaviour, which includes cytology, nutrition, and metabolism, (2) massive clonal propagation of



genotypes of interest, for rapid production of a large number of uniform plants, (3) production and propagation of disease-free plants by meristem culture, (4) induction of somaclonal variation, to obtain new genotypes, (5) management and modification of the ploidy levels, (6) embryo rescue, to recover hybrid plants that do not produce fertile seeds, (7) germplasm storage, for plant conservation or for cryopreservation, (8) genetic transformation, for the development of transgenic plants with resistance against diseases and environmental stresses, and with improved nutritional quality, and (9) production of compounds of pharmaceutical and industrial interest (García-González *et al.*, 2010; (Thorpe, 2013).

#### **1.2.6 Plant regulatory mechanism in plant tissue culture**

Plant pluripotent stem cells reside in meristems. Two primary meristems, the shoot apical meristem and the root apical meristem, are responsible for plant longitudinal growth and are located at the tip of the stem and root, respectively. In addition, plants develop a secondary meristem, the cambium, that allows them to grow radially, and which contributes cells to their vasculature and for mechanical support structures. The control of meristematic activity is therefore crucial for allowing plants to establish their body plan, maintain tissue homeostasis and adapt their development to fluctuating environments (Gaillochet and Lohmann, 2015).

Early studies on plant tissue culture and regeneration from callus defined crucial roles for the plant hormones auxin and cytokinin in coordinating cell divisions and cell differentiation. These hormonal pathways involved in many developmental processes, ranging from setting up the plant body plan to responding to environmental stresses. Accordingly, maintaining the balance between auxin and cytokinin is a key feature of

plant stem cell regulation, both to maintain stem cell activity and to define the functional domains guiding stem cell differentiation (Gaillochet and Lohmann, 2015).

In the shoot apical meristem, phytohormonal pathways are integrated into local transcriptional networks and control the level of *WUSCHEL* (*WUS*) expression, which subsequently regulates shoot stem cell activity. The cytokinin signalling domain encompasses the organizing centre and the neighbouring cell layers, where cytokinin integrates *WUS*. *WUS* sensitizes the organizing centre to cytokinin signalling by directly repressing the expression of type-A *ARABIDOPSIS RESPONSE REGULATORS* (*ARRs*), which are negative regulators of cytokinin signalling, and thereby promotes its own expression (Gaillochet and Lohmann, 2015).

Auxin synergizes with cytokinin to promote stem cell activity in the centre of the meristem. To balance organ formation with stem cell activity, auxin signals through the *AUXIN RESPONSE FACTOR* (*ARF*) transcription factor *MONOPTEROS* (*MP*), which directly represses the expression of *ARR7* and *ARR15*, thereby enhancing cytokinin signalling output and thus *WUS* expression (Zhao *et al.*, 2010).

In contrast to cytokinin which promotes proliferation, auxin signalling guides primordium initiation, leading to the formation of lateral organs, leaves or flowers. The regular arrangement of lateral organs along the main plant axis (phyllotaxis) is regulated mainly by a feed-forward system, involving the intercellular transport of auxin by the *PINFORMED1* (*PIN1*) auxin efflux carrier and auxin concentration or flux, which in turn impinges on *PIN* expression and localization (Gaillochet and Lohmann, 2015).

### 1.2.7 Micropropagation

The heterozygous nature of most angiosperms has made their seed progeny not true-to-type. Asexual reproduction, however, gives rise to plants that are genetically identical to the parent plant, hence permitting the perpetuation of the unique characters of the cultivars. One of the alternative means of asexual reproduction used today is micropropagation. Micropropagation is a plant tissue culture method for plant propagation, in which tissue is taken from a plant and grown on a nutrient medium in a laboratory to produce plantlets that are genetically identical to the parent (*The American heritage dictionary of the English language*, 2011). An aggregate of genetically identical plants asexually produced from a single plant constitutes a clone (*Merriam-Webster dictionary*, 2017).

The most significant advantage offered by the micropropagation methods over the conventional propagation methods is that in a relatively short time and space, a large number of plants that are genetically true-to-type can be produced starting from a single individual. Micropropagation is also useful for plants that are difficult to propagate by conventional methods, for example, plants producing little or no viable seeds. Through micropropagation, production of plants can be continued all the year round, independent of seasonal changes. Moreover, micropropagation methods are available to free plants from specific virus diseases (George *et al.*, 2008).

There are five stages fundamental to successful micropropagation of plants. The stages are stage 0 (selection and preparation of donor plant), stage I (establishment of aseptic culture), stage II (production of suitable propagules), stage III (preparation for growth in the natural environment), and stage IV (transfer to the natural environment) (Sathyanarayana and Varghese, 2007).

Stage 0 involves the selection and preparation of donor plant. It is essential to give careful attention to the selection of a mother plant, which is typical of the variety and disease-free. Success in the *in vitro* establishment depends on the physiological and phytosanitary qualities of the mother plant (Sathyanarayana and Varghese, 2007). Growth, morphogenesis, and propagation rate can be improved by appropriate environmental and chemical pre-treatment of the mother plants. To increase the probability of success, it is suggested that during this stage, the plants used as explant donors should be cultivated under optimal conditions, with irrigation, nutrition and temperature control (Cassells and Doyle, 2005). Fungicide, bactericide and insecticide treatments can be applied to diminish the level of infestation by insects and infection, either by microorganism infecting the plant or by endophytic microorganisms that circulate throughout the tissues (García-González *et al.*, 2010).

Stage I is the establishment of aseptic culture. This stage is crucial in order to obtain an aseptic culture of the selected plant material. The introduction of plant tissues into an *in vitro* culture is carried out through surface-sterilization with disinfectants, commonly sodium hypochlorite, calcium hypochlorite, and ethanol (García-González *et al.*, 2010). Nevertheless, some tissues with high lignin or cellulose content, such as woody plants and tissues of organs developed in the soil, need more drastic disinfection treatments such as short immersion in mercuric (II) chloride ( $\text{HgCl}_2$ ) (Husain and Anis, 2009). The surface-sterilized explants should be isolated to culture medium under sterile conditions, usually in a laminar flow cabinet. There is no single medium can be suggested as being entirely satisfactory for the growth of all types of plant tissues and organs; however modifications of MS basal medium (Murashige and Skoog, 1962) are most frequently used. Carbon source, such as sucrose, glucose, and fructose is also essential to the culture medium. In addition to the nutrients, it is necessary to add one or

more growth hormones, such as auxins, cytokinins and gibberellins to support good growth of tissues and organs. The culture medium can be made either solid with the addition of agar, or liquid without agar. Environmental culture conditions like light, temperature, and relative humidity must be controlled (Sathyanarayana and Varghese, 2007). A plant tissue is considered to be “established” to the *in vitro* culture when explants are not only free from superficial or visible contaminants, which interfere with the morphogenic response, but also when they show a morphogenic response, like multiplication and/or differentiation of the plant tissues such as shoots, roots, leaves or production of callus (García-González *et al.*, 2010).

Next is stage II, the production of suitable propagules. The aim of this stage is to bring about the multiplication of organs and structures that are able to give rise to plantlets until the desired number is obtained (Sathyanarayana and Varghese, 2007). Broadly, three approaches have been followed to achieve *in vitro* shoot propagation. The first one is through callusing, where the differentiation of plants from cultured cells may occur via shoot-root formation or somatic embryogenesis; the second approach is through adventitious bud formation, where the buds arise from any place other than leaf axil or shoot apex, for example root, leaf and stem cuttings; and the third approach is through axillary branching, where the buds develop into shoots from leaf axil or shoot apex (Bhojwani and Razdan, 1983). Shoots produced at this stage can be thought of as propagules in that they can usually be cultured again to increase their number. The multiplied shoot clusters may be divided into single or small group of shoots and transferred to fresh medium for further proliferation. To encourage repeated enhanced shoot production, it is necessary to study and establish the effect of plant growth regulators (García-González *et al.*, 2010). Generally, cytokinin as BAP and kinetin, alone or in interaction with a lower level of auxin as IBA and NAA causes shoot

proliferation; while 2,4-D is effective for the induction and growth of callus (Bhojwani and Dantu, 2013).

Stage III refers to the preparation for growth in the natural environment. This stage involves shoot elongation and rooting of the shoots prior to their transfer to soil. Although root formation may occur simultaneously to propagation in the same culture media used for shoot multiplication, however, in some cases, it is first necessary to grow elongated shoots ready for rooting and then utilise special media to induce root formation (Sathyanarayana and Varghese, 2007). For rooting treatment, individual shoots may be transferred to the rooting media with or without growth hormone, usually auxin. Alternatively, the shoots may be rooted *ex vitro* by transferring the cuttings directly to the soil (George *et al.*, 2008).

The last stage is stage IV, which is the transfer to the natural environment. This stage is referred to as acclimatization. Acclimatization is the process by which the plantlets physiologically and anatomically adjust from *in vitro* to *ex vitro* conditions (Sathyanarayana and Varghese, 2007). Acclimatization is important for better survival of plantlets to the natural environment. Management of light intensity, substrate characteristic, substrate moisture, and temperature at the leaf and root level are suggested, because they can influence the general physiological characteristics of the plants (García-González *et al.*, 2010). At the beginning of the adaptation phase, a constant high relative humidity is recommended to facilitate the formation of active roots and to reduce water lost by transpiration, but as plants start to adapt, it is recommended to decrease the humidity in order to facilitate a better adaptation to field conditions (García-González *et al.*, 2010).

### 1.2.8 Coloured callus formation

Having high plasticity for cell differentiation is one characteristic of plant cells. Plants generate unorganized cell masses, such as callus or tumors, in response to stresses, such as wounding or pathogen infection. In biotechnology, a mass of undifferentiated cells produced as the first stage in tissue culture is called callus (*Collins English dictionary*, 2012). The balance between auxin and cytokinin promotes callus induction in various plant species. Callus can be produced from a single differentiated cell, and many callus cells are totipotent, being able to regenerate the whole plant body. Under certain conditions, callus cells also undergo somatic embryogenesis, a process in which embryos are generated from adult somatic cells (Ikeuchi *et al.*, 2013).

Callus can be classified into subgroups based on their macroscopic characteristics. For example, callus with no apparent organ regeneration typically are called compact or friable callus. In compact callus, the cells are densely aggregated, while in friable callus, the cells are loosely associated with each other and the callus is soft and breaks apart easily (Slater, 2008). Other callus that display some degrees of organ regeneration are called rooty, shooty, or embryonic callus, depending on the organs they generate (Ikeuchi *et al.*, 2013).

Callus cultures can also be of different colours. The colours are determined by the type and combination of pigments present in the callus. Pigment is an organic compound that gives a characteristic color to plant or animal tissues and is involved in vital processes (*The American heritage student science dictionary*, 2014). Generally, pigments are classified into four major groups: (1) chlorophylls (e.g. chlorophylls), (2) carotenoids (e.g. carotenes and xanthophylls), (3) flavonoids (e.g. anthocyanins, aurones, chalcones, flavonols, and proanthocyanidins), and (4) betalains (e.g.

betacyanins and betaxanthins). Chlorophylls exhibit green colour; carotenoids consist of two sub-groups, carotenes which are yellow, orange, and red in colour, and xanthophylls which are yellow in colour; flavonoids are classified into about a dozen groups, such as anthocyanins (orange, red, purple, and blue), aurones (yellow), chalcones (yellow), flavonols (cream), and proanthocyanidins (beige, tan, red, brown, and black); and betalains are split into orange and red betacyanins and yellow betaxanthins (Davies, 2004; Tanaka *et al.*, 2008). Studies on natural pigments have become popular nowadays because these phytochemicals are rich in nutrients and may provide benefits to human health. Natural pigments can be used commercially in many fields including agriculture, medicine, cosmetic, and food industry, since artificial colourants are found to have negative effects on health (Elias *et al.*, 2015).

Production of callus from fragments of stems, leaves, and roots are mainly carried out to determine the culture conditions required by the explants to survive and grow, study cell development, exploit products coming from primary and secondary metabolism, and obtain cell suspension in propagation (Sen *et al.*, 2014). It can also pave the way for isolating economically valuable phytochemicals, which can avoid collecting plant materials from natural sources. In the area of plant biotechnology, callus and cell culture carries a special role for producing medicinal and bioactive compounds in large-scale from plants (Sen *et al.*, 2014).

Various plant species have been used for the establishment of callus and cell cultures for phytochemical production. Phytochemical production in callus cultures of a species relies on synergistic effects of plant growth regulators, carbon source, pH, and light. Sreenivas *et al.* (2011) demonstrated that the callus of *Bridelia stipularis* incubated in light on MS medium supplemented with BAP and 2,4-D plus 4% glucose



at pH 3.5 yielded the highest amount of anthocyanins. Plant growth regulator have a major effect on coloured callus induction. Elias *et al.* (2015) observed different colours of callus in stem cultures of *Echinocereus cinerascens*, with the highest percentage of green callus was obtained on MS medium supplemented with BAP, yellow callus on MS medium with NAA and BAP, and pink callus on MS medium with NAA. In some species, the type of explants influences the colour of callus produced. For example, in *Achyranthes aspera*, when cultured on MS medium supplemented with 2,4-D and NAA, the leaf explants produced green callus, while internode explants produced purple callus, and root explants produced white callus (Sen *et al.*, 2014).

### **1.2.9 *In vitro* flowering**

Flowering involves the conversion of apical meristematic initials into a floral meristem, from which all the parts of the flower will be produced (Murthy *et al.*, 2012). The ability of explants to form flowers *in vitro* depends on numerous factors, internal and external, chemical and physical, and virtually all of these factors interact in various complex and unpredictable ways (Sudhakaran and Sivasankari, 2002). A number of plant species have been reported to produce *in vitro* flowering. These include ornamental plants like orchid *Dendrobium Chao Praya Smile* (Hee *et al.*, 2007) and rose (Vu *et al.*, 2006), commercial crops like bamboo (Lin *et al.*, 2004), medicinal plants like *Vitex negundo* (Thiruvengadam and Jayabalan, 2001), food crops like green pea (Franklin *et al.*, 2000), and rare and endangered plants like *Rauvolfia tetraphylla* (Sarma *et al.*, 1999).

Several reports have demonstrated that there is considerable variability in the requirements of plant growth regulators, temperature, light regime, and nutritional factors for *in vitro* flower development in explants from different species (Murthy *et al.*,

2012) and experimental evidence supports a multiplicity of factors regulating the *in vitro* flowering process. For instance, in wild mustard (*Cleome viscosa*), *in vitro* flowers developed best on half-strength MS medium with BAP and IBA combination and 4% sucrose (Rathore *et al.*, 2013), while in green pea (*Pisum sativum*), maximum *in vitro* flowering was obtained on MS medium with half strength ammonium nitrate, GA<sub>3</sub> and IBA combination, and 3% sucrose (Franklin *et al.*, 2000). The age of cultures also influences the development of *in vitro* flowers (Franklin *et al.*, 2000).

Flowering plantlets *in vitro* has a good commercial potential for ornamental trade (Wang *et al.*, 2002; Zeng *et al.*, 2013). An *in vitro* flowering mechanism is considered to be a convenient tool to study specific aspects of flowering and whole mechanisms of the reproductive process such as floral initiation, floral organ development, and floral senescence (Murthy *et al.*, 2012). There is also a possibility of using *in vitro* flowering in ecological and genetic studies (Sivanesan and Jeong, 2007). *In vitro* flowering bears importance in selective hybridization especially in using pollen from rare stocks and may be the first step towards the possibility of recombining genetic material via *in vitro* fertilization in otherwise non-hybridizable lines (Murthy *et al.*, 2012). Early *in vitro* flowering shortens the juvenile phase (Hee *et al.*, 2007) and the breeding cycle to generate better quality of plant varieties that can meet the market demand (Haque and Ghosh, 2013). *In vitro* flowering can also be adjusted to the commercial production of specific compounds from floral organs (Zeng *et al.*, 2013).

#### **1.2.10 Assessment of pollen and seed quality**

A successful fertilization for seed and fruit formation greatly depends on the quality of pollen grains. Viable pollen can be defined as pollen that is competent to deliver two male gametes to the embryo sac (Heslop-Harrison, 1992). Methods to assess

pollen quality aim to find the capacity of pollen to deliver gametes. Pollen quality is normally measured by looking at viability and germinability of the pollen.

Pollen viability is usually estimated by staining of pollen grains with dyes, such as acetocarmine and 2,3,5-triphenyltetrazolium chloride (TTC). Acetocarmine test, which stains pollen nuclei, determines the chromatin integrity of pollen. The pollen stained with acetocarmine take on a reddish hue when viable, and remains transparent when non-viable. In turn, the test with TTC indicates the presence of dehydrogenase enzymes by means of the red coloration of viable pollen. The pollen grains that were not colored were considered non-viable (Soares *et al.*, 2016)

*In vitro* pollen germination test determines the actual germination capability of pollen under suitable conditions (Tuinstra and Wedel, 2000). This test built on the assumption that pollen that germinates and produces a tube *in vitro* is likely to do so *in vivo*, and to fertilize the egg. Germination capability of pollen depends on various factors, namely, nutrition conditions, species or variety used, and environmental factors (Gaaliche *et al.*, 2013). Many pollen grains can germinate in water or aqueous solutions of sucrose with no additives, but pollen of some species needs special substrates for germination (Beyhan and Serdar, 2008). The sucrose used in the germination medium aims to provide a balance between the osmotic solution and pollen germination, and to provide energy to assist the process of development of pollen tubes (Lyra *et al.*, 2011). The pollen germination results can also be used as an indicator of pollen viability.

Pollen quality needs to be assessed to find plant fertility and to monitor pollen state during storage, since pollen must be viable at the time of pollination for seed or fruit set to occur. The pollen staining and germination tests allow measurement of

pollen viability by examining and measuring cellular features characteristic of living cells, and they indicate reasons for inviability, hence enable manipulation to overcome the problem (Heslop-Harrison, 1992). Information on pollen viability and germinability is important for the study of reproductive biology and for the development of genetic crop programmes, as it enables a greater success rate of crosses (Lyra *et al.*, 2011).

The embryo, contained within the seed, is the next generation of a plant. Thus, successful seed germination is vital for a species to perpetuate itself. Seed germination is the process by which the embryo wakes up from the state of dormancy and takes to active life (Gupta, 2016). This process covers all the changes from the earliest sprouting of the seed till it establishes itself as an independent plant. A seed germination test determines the maximum germination potential, or viability, of the seed (20/20 Seed Labs, 2010). The germination rate (percentage) of a particular seed lot is a key indicator as to how that seed will perform in the field. Conditions such as oxygen, light, moisture and temperature need to be standardized for every plant type to ensure that germination will take place within a specific period of time.

In general, seed germination results were used for the following purposes: (1) to determine seed quality, that is, its suitability for planting, (2) to identify seed quality problem and its probable cause, (3) to determine the need for drying, processing, or specific procedures that should be used to overcome the seed quality problem, (4) to clarify if seed meets the established quality standards or labelling specifications, and (5) to establish quality and provide a basis for price and consumer discrimination among lots in the market (TNAU, 2014). Information on seed germinability is important for helping to determine whether a particular seed lot has the potential to produce a good plant, or should be used at all (20/20 Seed Labs, 2010).

### 1.2.11 Ultrastructure of non-glandular and glandular trichomes

Ultrastructure is defined as “the detailed structure of a biological specimen, such as a cell, tissue, or organ that can be observed only by electron microscopy” (*The American heritage dictionary of the English language*, 2011). A scanning electron microscope (SEM) is a type of electron microscope that utilizes a focused beam of high-energy electrons at the surface of solid specimens to produce high resolution images. The interaction between electron and sample generates a variety of signals that reveal information about the sample including external morphology, topography, chemical composition, and crystalline structure and orientation of materials making up the sample (Swapp, 2017).

Biological specimens require prudent preparation procedures to obtain good images in normal high vacuum SEM systems. Standard sample preparation procedures for SEM involve chemical fixation, dehydration/drying, mounting on a stub, and coating with a metal (Pathan *et al.*, 2008). The fixation and dehydration/drying steps need to be done as carefully as possible to reduce shrinkage while ensuring preservation of the structures of cells, tissues, or organisms as close to the natural state as possible. Chemical fixation is performed by soaking the sample in a chemical fixative formulation, usually glutaraldehyde and/or osmium tetroxide. The samples may be subsequently dehydrated in a graded series of organic solvents such as ethanol and acetone, and then dried by critical point drying (CPD) with the use of a transitional fluid such as liquid carbon dioxide. This procedure removes liquids from the specimen and avoids surface tension effects (drying artefacts) by never allowing a liquid–gas interface to develop within the sample during drying (Pathan *et al.*, 2008). The dried sample is usually mounted on an SEM stub using a double-sided carbon adhesive, and sputter-coated with a thin layer of metal, like gold, gold-palladium, platinum, chromium,

iridium, and carbon, to increase electron conductance on the sample, before examination in the SEM (Murtey and Ramasamy, 2016).

Ultrastructural studies of non-glandular and glandular trichomes by SEM provide useful information on the structures and functions of the trichomes in the plant species. Glandular trichome ultrastructural study can reveal the morphology, types, ontogeny, and pattern of distribution of the glandular trichomes in the leaves and flowers of *Leonotis leonurus* at different stages of development (Ascensão *et al.*, 1995). Ioannidis *et al.* (2002) were able to study the effects of UV-B treatment on the shape, volume, and number of glandular trichomes in *Ocimum basilicum* through their scanning electron micrographs. Qualitative and quantitative correlations between glandular trichomes and essential oils secretion have also been studied. The morphology and distribution of non-glandular trichomes on leaf and flower surfaces have brought to the knowledge about their function as protection against dryness (Werker, 2000).

#### **1.2.12 Extraction and analysis of essential oil content**

An essential oil is a volatile oil derived from some parts of a plant, for example the flowers, leaves, stems, roots, seeds, barks, or fruit rinds, and usually carries the odour or flavour of the plant from which it is extracted (*The American heritage dictionary of the English language*, 2011). Plants store essential oils either in external secretory structures called glandular trichomes, which are found on the surface of the plant, or internal secretory structures called secretory cavities and ducts, which are found inside the plant material. Plants produce essential oils for a variety of purposes: (1) to attract pollinators and dispersal agents, (2) to play a role in allelopathy, to prevent competing plant species from growing around them, (3) to serve as defence compounds

against insects and herbivores, and (4) to act as antifungal and antibacterial agents against microorganisms that may threaten the survival of the plants (NAHA, 2016).

Essential oils contain various aromatic compounds that influence the psychological, spiritual, and physical aspects of the human body. The diluted oils may be inhaled, applied topically, and in some cases ingested, to promote relaxation, increase feelings of well-being, reduce stress, relieve pain, treat infections, promote healing, boost immune system, and fight inflammation (Dylanna Press, 2014). When an essential oil is inhaled, its scent is carried to the brain, where the limbic centre is stimulated to release neurotransmitters such as serotonin and dopamine, as well as endorphins. Depending upon the essential oil inhaled, one may feel aroused, excited, or relaxed. Essential oils also work by being absorbed through the skin into the blood stream. They can be added to carrier oils, lotions, and other ointments and massaged into the skin to relieve tired muscles, pains, aches, and a variety of skin conditions (Dylanna Press, 2014).

Essential oils can be extracted via two key methods, namely distillation and expression. Absolutes, highly concentrated aromatic substances, on the other hand, can be extracted via solvent extraction or enfleurage. Carbon dioxide (CO<sub>2</sub>) extracts, can be extracted via supercritical CO<sub>2</sub> extraction (NAHA, 2017).

Distillation is most often employed with the extraction of essential oil from leaves and flowers (rose and orange blossoms). During distillation, the plant material is placed upon a grid inside the still, and the still is sealed. Depending upon the method used, steam or water–steam slowly breaks through the plant material to remove its volatile constituents. These volatile constituents rise upward through a connecting pipe

that leads them into a condenser. The condenser cools the rising vapor back into liquid form. The liquid is then collected in a vehicle below the condenser. The essential oil can be found on the surface of the water where it is siphoned off. Occasionally, an essential oil is heavier than water and is found on the bottom rather than the top, such as with clove essential oil (NAHA, 2017). There are three types of distillation for isolating essential oils from plant materials: (1) water distillation – the plant material comes into direct contact with the water, (2) water and steam distillation – the water remains below the plant material, which has been placed on a grate while the steam is introduced from outside the main still (indirect steam), and (3) direct steam distillation – the plant material is supported on a grate while the steam is injected into the still (NAHA, 2017).

Expression, also referred to as cold pressing or *ecuelle a piquer*, is a method of extraction specific to citrus essential oils, such as lemon, bergamot, orange, and lime. During this process, the rind of the fruit is placed in a container having spikes that puncture the peel while the device is rotated. The puncturing of the rind releases the essential oil that is then collected in a small area below the container. The spinning in a centrifuge separates the essential oil from the fruit juice (NAHA, 2017).

Solvent extraction and enfleurage are employed as methods of flower scent extraction from delicate flowers like jasmine, tuberose, and carnation. Solvent extraction is the use of solvents, such as petroleum ether, methanol, ethanol, or hexane, to extract the odoriferous lipophilic materials from the plants. Enfleurage, on the other hand, is a cold-fat extraction process that uses fat to absorb the plant aromatic substances. The first product made via solvent extraction and enfleurage is known as a concrete, which contains the plant waxes, fats, and odoriferous materials. The concrete



is then mixed with alcohol to extract the aromatic principle of the flowers. The final product is known as an absolute (NAHA, 2017).

Supercritical CO<sub>2</sub> extraction is a relatively new process used for the extraction of aromatic products. The basic concept is that CO<sub>2</sub> under pressure will turn from a gas into a liquid that can then be used as an inert liquid solvent. This liquid solvent is able to diffuse throughout the plant material thus extracting its aromatic constituents. CO<sub>2</sub> extracts are known for their strong similarity in aroma to the actual plant aroma (NAHA, 2017).

Gas chromatography mass spectrometry (GC–MS) is an instrumental technique, comprising a gas chromatograph (GC) coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identified and quantified (Bull, 2008). In order for a compound to be analysed by GC–MS, it must be sufficiently volatile and thermally stable. GC–MS analysis is in wide use for confirmation testing of substances, such as in drug testing, manufacturing quality control, and environmental testing (Douglas, 2010).

GC analysis separates all of the components in a sample and provides a representative spectral output. The sample solution is injected into the GC inlet where it is vaporized and swept onto a chromatographic column by a carrier gas, usually helium. The sample flows through the column and the compounds comprising the sample are separated by virtue of their relative interaction with the coating of the column (stationary phase) and the carrier gas (mobile phase). The compounds then elute from the column separately. The time elapsed between injection and elution is called the “retention time”. Each component ideally produces a specific spectral peak (Bull, 2008).

MS identifies substances by electrically charging the specimen molecules, accelerating them through a magnetic field, breaking the molecules into charged fragments and detecting the different charges. The MS instrument produces the output by drawing an array of peaks on a chart, the “mass spectrum”. Each peak represents a value for a fragment mass (Douglas, 2010).

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**Figure 1.1:** Intact plant of *O. basilicum*.



**Figure 1.2:** Seeds and fruiting calyx of *O. basilicum*.

### 1.3 Research Objectives

This study aimed to develop efficient micropropagation protocols of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol for mass production of coloured callus, plants, and flowers containing high levels of uniformity in the aspects of pollen and seed germinability, non-glandular and glandular trichome ultrastructure, and essential oil content and composition.

In order to achieve the aim, several steps need to be taken. The specific objectives of this research were:

1. to identify the optimum PGR or combination of PGRs that would best support induction of coloured callus formation from leaf and shoot tip explants of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol for production of coloured callus.
2. to identify the optimum PGR or combination of PGRs that would best support induction of shoot multiplication from leaf and shoot tip explants, and to measure the efficacy of half-strength MS basal medium to promote *in vitro* rooting from multiple shoots of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol for production of complete *in vitro* plantlets.
3. to identify the optimum PGR or combination of PGRs that would best support induction of *in vitro* flowering from leaf and shoot tip explants, and to measure its efficacy to induce *in vitro* flowering from multiple shoots of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol for production of normal flowers.

4. to measure the efficacy of vermiculite, followed by a soil mixture of three parts garden soil to two parts potting mix to promote acclimatization of *in vitro* regenerated plants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol for establishment and flowering of the plants under *ex vitro* conditions.
5. to compare the germination rate of pollen grains and seeds between the *in vitro*, *ex vitro*, and *in vivo* grown plants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol for establishment of information on pollen and seed germinability of the plants at different developmental stages.
6. to compare the distribution, shape, and density of non-glandular and glandular trichomes on leaves and flowers between the *in vitro* and *in vivo* grown plants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol for better understanding on the relationship between their morphology and the plant developmental stages.
7. to compare the essential oil content and composition in leaves between the *in vitro*, *ex vitro* and *in vivo* grown plants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol for establishment of information on essential oil and compound accumulation of the plants at different developmental stages.

## CHAPTER 2: COLOURED CALLUS FORMATION OF *Ocimum basilicum* L.

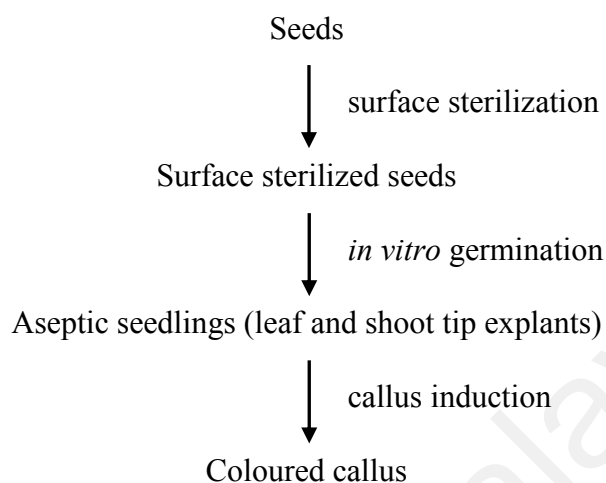
### 2.1 Objectives of the Experiment

Callus is an actively dividing non-organized mass of cells that develops on or around a wounded or cut plant surface. Coloured callus usually contains plant pigments which are biologically active. In plant tissue culture, actively growing callus can be initiated from plant parts by the addition of PGRs to plant tissue culture media. The extent of overall callus formation and pigments accumulation usually depend on the cytokinin and auxin balance of the plant tissue culture media and the type of the plant materials (explants) used.

In this chapter, the objective of the experiment was to identify the optimum PGR or combination of PGRs that would best support induction of coloured callus formation from leaf and shoot tip explants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol for production of coloured callus. In this experiment, leaf and shoot tip explants from two-month-old aseptic seedlings of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol were cultured on culture media containing MS medium, 3% sucrose, and 0.8% agar at pH 5.8; and different combinations and concentrations of PGRs were tested for maximum coloured callus formation from the explants.

## 2.2 Materials and Methods

### 2.2.1 Experimental outline



### 2.2.2 Source of explants

In this work, *Ocimum basilicum* L. 'Sweet Thai', chemotype methyl chavicol was used as the reference mother plant. Its morphological and aromatic characteristics were as follows: herb; compact; purple, branching, square stems; green, narrowly ovate, gland-dotted leaves; purple spikes; white with purple tinge, bilabiate with a lower lip and a four-lobed upper lip, funnel-shaped flowers; black, ovoid, mucilaginous when wet seeds; and sweet, anise-like, slightly spicy aroma (Paton, 1992; Simon *et al.*, 1999; Raghavan, 2006).

Mature seeds of *O. basilicum* L. 'Sweet Thai', chemotype methyl chavicol, borne in the fruiting calyx of the plant, were collected from a single mother plant in a village garden in Perak, Malaysia and germinated under *in vitro* conditions. Later, the two-month-old aseptic seedlings were used as the source of leaf and shoot tip explants to initiate *in vitro* cultures.

### **2.2.3 *In vitro* germination of seeds**

#### **2.2.3.1 Preparation of germination media**

To prepare 1 l of germination medium (MS basal medium), 30 g of sucrose (Duchefa Biochemie B.V., Haarlem, The Netherlands) followed by 4.4 g of MS powdered medium (Duchefa Biochemie B.V., Haarlem, The Netherlands) were dissolved in 700 ml of distilled water in a 1-l conical flask. The medium solution was homogenized using a magnetic stirrer on a stirring hot plate. Then, the volume of the medium was brought up to 1 l with distilled water in a 1-l graduated cylinder, and the pH of the medium was adjusted to  $5.8 \pm 0.1$  using 1.0 N hydrochloric acid (HCl) and/or 1.0 N sodium hydroxide (NaOH). After that, the medium was gelled with 8 g of agar technical (Oxoid Ltd., Basingstoke, Hampshire, England). The medium was distributed in 200-ml glass jars, with each glass jar contained approximately 45 ml of the medium. The medium-containing glass jars were capped with plastic caps and autoclaved at 121°C and 15 psi for 20 minutes.

#### **2.2.3.2 Surface sterilization of seeds**

Before germinating seeds on a germination medium, the seeds need to be surface sterilized to remove impurities and microbial contaminants. Firstly, the seeds were washed three times with distilled water, each time for 5 minutes. This was done by shaking the tightly-capped specimen container filled with the seeds and distilled water. After that, the seeds were surface sterilized with a series of Clorox bleach (5.25% sodium hypochlorite; The Clorox Company, Oakland, California, USA) dilutions and then ethanol, in steps as listed below:

- (1) Seeds were treated with 70% Clorox plus 0.1% Tween-20 for 6 minutes.
- (2) Seeds were treated with 40% Clorox for 6 minutes.
- (3) Seeds were treated with 20% Clorox for 6 minutes.



- (4) Seeds were rinsed with sterile distilled water.
- (5) Seeds were immersed in 70% ethanol for 1 minute.
- (6) Seeds were rinsed five times with sterile distilled water to remove traces of the sterilizing agents.

All the surface sterilization procedures starting from the Clorox treatment were conducted in a Microflow laminar flow cabinet (MDH Ltd., Andover, Hampshire, England) to maintain the aseptic conditions.

### **2.2.3.3 *In vitro* germination of seeds**

Three to four surface sterilized seeds per glass jar were placed aseptically on the germination media using a sterile forceps. The glass jars were capped tightly and sealed with parafilm. All seed cultures were incubated in a growth chamber maintained at  $25 \pm 2^\circ\text{C}$  under 16 hours of light and 8 hours of darkness, with an irradiance of  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  provided by Philips Lifemax TL-D 18W cool daylight fluorescent tubes (Royal Philips, Amsterdam, The Netherlands). The percentage of seed germination was recorded 21 days after germination.

## **2.2.4 Induction of coloured callus formation**

### **2.2.4.1 Preparation of callus induction media**

Callus induction media were prepared using procedures as described in Section 2.2.3.1. One litre of medium consisted of 4.4 g of MS medium, 30 g of sucrose, and 8 g of agar at pH  $5.8 \pm 0.1$ . The difference was that the callus induction medium was added with different combinations of PGRs such as 6-benzyl-aminopurine (BAP; Sigma-Aldrich Co., St. Louis, Missouri, USA),  $\alpha$ -naphthaleneacetic acid (NAA; Sigma-Aldrich Co., St. Louis, Missouri, USA), 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma-Aldrich Co., St. Louis, Missouri, USA), and gibberellic acid ( $\text{GA}_3$ ; Sigma-Aldrich Co., St.

Louis, Missouri, USA) to induce coloured callus formation. BAP and NAA were dissolved in dilute NaOH, while 2,4-D and GA<sub>3</sub> were dissolved in distilled water. BAP, NAA and 2,4-D were sterilized by autoclaving and added to the medium before the PGR and medium mixture was autoclaved, while GA<sub>3</sub> which is thermolabile was sterilized by membrane filtration and added to the autoclaved medium when the medium had cooled to around 50°C. For membrane filtration of GA<sub>3</sub>, GA<sub>3</sub> solution carried in a graduated syringe was gradually pushed through an Acrodisc syringe filter with 0.2 µm Supor membrane (Pall Corp., Ann Arbor, Michigan, USA) and the sterilized GA<sub>3</sub> solution dripping out from the membrane filter was collected in a sterile container. The callus induction medium was distributed in 60-ml PS sterile specimen containers, with each container was filled one-quarter full (≈15 ml) with the medium. PGR-free MS medium was used as a control. The PGR combinations and concentrations tested were as listed below:

- (1) MS basal medium (no PGR)
- (2) MS medium + 0.5 mg l<sup>-1</sup> BAP
- (3) MS medium + 1.0 mg l<sup>-1</sup> BAP
- (4) MS medium + 1.5 mg l<sup>-1</sup> BAP
- (5) MS medium + 2.0 mg l<sup>-1</sup> BAP
- (6) MS medium + 0.5 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> NAA
- (7) MS medium + 1.0 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> NAA
- (8) MS medium + 1.5 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> NAA
- (9) MS medium + 2.0 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> NAA
- (10) MS medium + 0.5 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> NAA
- (11) MS medium + 1.0 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> NAA
- (12) MS medium + 1.5 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> NAA
- (13) MS medium + 2.0 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> NAA

- (14) MS medium + 0.5 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> 2,4-D
- (15) MS medium + 1.0 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> 2,4-D
- (16) MS medium + 1.5 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> 2,4-D
- (17) MS medium + 2.0 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> 2,4-D
- (18) MS medium + 0.5 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> 2,4-D
- (19) MS medium + 1.0 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> 2,4-D
- (20) MS medium + 1.5 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> 2,4-D
- (21) MS medium + 2.0 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> 2,4-D
- (22) MS medium + 0.5 mg l<sup>-1</sup> GA<sub>3</sub>
- (23) MS medium + 1.0 mg l<sup>-1</sup> GA<sub>3</sub>
- (24) MS medium + 1.5 mg l<sup>-1</sup> GA<sub>3</sub>
- (25) MS medium + 2.0 mg l<sup>-1</sup> GA<sub>3</sub>

#### **2.2.4.2 Induction of coloured callus formation**

Two types of explants were used in this experiment, which are the leaves and shoot tips from two-month-old aseptic seedlings. Using a sterile scalpel and forceps, the leaf and shoot tip explants were cut into uniform sizes. For leaf explants, the centres of the leaves were cut at in a square shape of approximately 5x5 mm<sup>2</sup> area. For shoot tip explants, the shoot tips were cut to approximately 7 mm length. Then, they were placed on the callus induction media, containing MS medium supplemented with different combinations of PGRs as listed in Section 2.2.4.1. The leaves were placed abaxial side down on the medium surface, while the shoot tips were placed vertically on the medium surface in sterile specimen containers. All shoot tip and leaf cultures were incubated in a growth chamber maintained at 25 ± 2°C under 16 hours of light and 8 hours of darkness, with an irradiance of 50 μmol m<sup>-2</sup> s<sup>-1</sup> provided by Philips Lifemax TL-D 18W cool daylight fluorescent tubes. The percentage of explants showing callus formation

and the intensity, colour, and texture of the callus formed were evaluated after 6 weeks of culture. The callus intensity was categorised into profuse (when the callus covered at least 75% of the cutting area, in large amount), moderate (when the callus covered 50% to 75% of the cutting area, in average amount), and poor (when the callus covered less than 50% of the cutting area, in small amount). The dominance of the coloured callus was categorised into extremely dominant (when the dominant colour covered at least 75% of the total callus area, with darker colour), highly dominant (when the dominant colour covered at least 75% of the total callus area, with lighter colour), and dominant (when the dominant colour covered 50% to 75% of the callus area, with lighter colour). Thirty explants per treatment were used as minimum large sample size ( $n \geq 30$ ), in order to obtain an approximately normal distribution of the sample means, according to the central limit theorem (LaMorte, 2016).

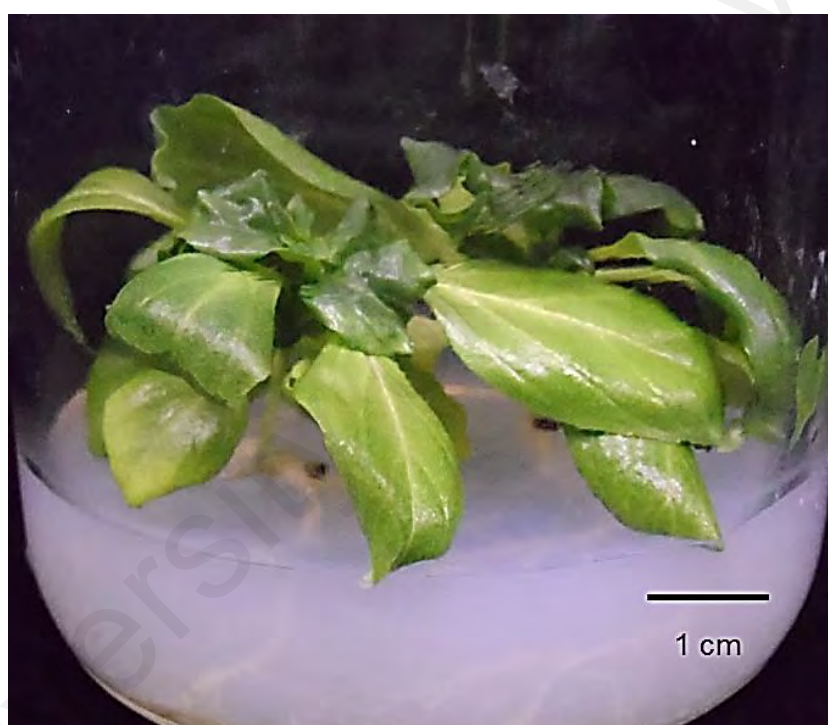
#### **2.2.5 Statistical analysis**

Data was statistically analysed using one-way analysis of variance (ANOVA) with confidence intervals calculated via Duncan's multiple range test (DMRT) at  $P = 0.05$ , using IBM Statistical Package for Social Science (SPSS) statistics version 22 software (IBM Corp., Armonk, New York, USA). Results were presented as mean  $\pm$  standard error (SE) of the mean.

## 2.3 Results

### 2.3.1 *In vitro* germination of seeds

Seeds of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol showed a 70% germination rate after 21 days of maintenance on MS basal medium. After two months, the aseptic seedlings (Figure 2.1) were used as the explant source to induce coloured callus formation.



**Figure 2.1:** Two-month-old aseptic seedlings of *O. basilicum* growing on MS basal medium.

### 2.3.2 Coloured callus formation from leaf explants

Coloured callus formation from leaf explants of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol were evaluated after 6 weeks of culture on the callus induction media. For leaf explants, MS medium without PGR which served as a control failed to induce any callus formation. MS medium supplemented with BAP alone and MS medium supplemented with GA<sub>3</sub> alone, at all concentrations tested were also not

effective in inducing callus formation from leaf explants. The leaves showed browning and eventually became necrosis.

MS medium supplemented with different combinations of cytokinin and auxin (BAP combined with NAA and BAP combined with 2,4-D) at all concentrations tested however, were effective to induce callus formation from leaf explants. The calluses were observed starting to form at the leaf vein cuttings after 14 days of culture (Figure 2.2). Two main colours were detected in the callus formed, which were purple and light green. Of the different media tested, the best media for the highest percentage of purple callus formation from leaf explants were MS medium supplemented with  $0.5 \text{ mg l}^{-1}$  BAP and  $0.5 \text{ mg l}^{-1}$  NAA and MS medium supplemented with  $1.0 \text{ mg l}^{-1}$  BAP and  $0.5 \text{ mg l}^{-1}$  NAA (Table 2.1). On these media, 100% of the explants produced callus, and the callus formed were profuse, compact, with purple callus showed high dominance over light green callus after 6 weeks of culture. On the other hand, MS medium supplemented with  $0.5 \text{ mg l}^{-1}$  BAP and  $1.0 \text{ mg l}^{-1}$  2,4-D and MS medium supplemented with  $1.0 \text{ mg l}^{-1}$  BAP and  $1.0 \text{ mg l}^{-1}$  2,4-D were the best media for the highest percentage of light green callus formation from leaf explants (Table 2.1). On these media, 100% of the explants produced callus, and the callus formed were profuse, semi-compact with light green callus showed extreme dominance over purple callus after 6 weeks of culture.

For BAP and NAA combination, 100% of the leaf explants formed callus at all concentrations tested. A low, equal balance of BAP and NAA concentration ( $0.5$  to  $1.0 \text{ mg l}^{-1}$  BAP combined with  $0.5 \text{ mg l}^{-1}$  NAA) favoured the formation of profuse, purple callus from the leaf explants (Figure 2.3). The callus developed on the BAP and NAA-supplemented medium were compact. At low concentration of NAA ( $0.5 \text{ mg l}^{-1}$ ),

increasing the concentration of BAP from 1.0 to 2.0 mg l<sup>-1</sup> resulted in reduced intensity of overall callus formation. The dominance of purple callus also decreased at BAP concentration higher than 1.0 mg l<sup>-1</sup>. At all BAP concentrations tested, a slight increase of NAA concentration from 0.5 to 1.0 mg l<sup>-1</sup> enhanced the formation of light green callus but reduced the dominance of purple callus.

For BAP and 2,4-D combination, 100% of the leaf explants formed callus at all concentrations tested, except for 2.0 mg l<sup>-1</sup> BAP combined with 0.5 mg l<sup>-1</sup> 2,4-D, and 2.0 mg l<sup>-1</sup> BAP combined with 1.0 mg l<sup>-1</sup> 2,4-D, in which 90% and 96.67% of the leaf explants, respectively, formed callus. A moderately low, equal balance of BAP and 2,4-D concentration (0.5 to 1.0 mg l<sup>-1</sup> BAP combined with 1.0 mg l<sup>-1</sup> 2,4-D) favoured the formation of profuse, light green callus from the leaf explants (Figure 2.4). The callus developed on the BAP and 2,4-D-supplemented medium were semi-compact to compact. At low concentration of 2,4-D (0.5 mg l<sup>-1</sup>), increasing the concentration of BAP from 1.0 to 2.0 mg l<sup>-1</sup> resulted in reduced intensity of overall callus formation. The dominance of light green callus also decreased at BAP concentration higher than 1.0 mg l<sup>-1</sup>. At all BAP concentrations tested, a slight increase of 2,4-D concentration from 0.5 to 1.0 mg l<sup>-1</sup> enhanced the formation and dominance of light green callus but suppressed the formation of purple callus.

**Table 2.1:** Coloured callus formation from leaf explants of *O. basilicum* cultured on MS medium supplemented with different PGRs, maintained at  $25 \pm 2^\circ\text{C}$  under 16 hours of light and 8 hours of darkness, with an irradiance of  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

| Concentration of PGRs ( $\text{mg l}^{-1}$ ) |           | Mean callus formation (%) $\pm$ SE <sup>x</sup> | Intensity of callus formation <sup>y</sup> | Colour <sup>z</sup>    | Texture      |
|--|-----------|---|--|------------------------|--------------|
| Cytokinin                                    | Auxin     |   |  |                        |              |
| 0  | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                      | -            |
| 0.5 BAP                                      | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                      | -            |
| 1.0 BAP                                      | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                      | -            |
| 1.5 BAP                                      | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                      | -            |
| 2.0 BAP                                      | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                      | -            |
| 0.5 BAP                                      | 0.5 NAA   | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple** & light green | Compact      |
| 1.0 BAP                                      | 0.5 NAA   | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple** & light green | Compact      |
| 1.5 BAP                                      | 0.5 NAA   | $100.00 \pm 0.00$ <sub>a</sub>                  | ++   | Purple* & light green  | Compact      |
| 2.0 BAP                                      | 0.5 NAA   | $100.00 \pm 0.00$ <sub>a</sub>                  | +  | Purple* & light green  | Compact      |
| 0.5 BAP                                      | 1.0 NAA   | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple & light green*  | Compact      |
| 1.0 BAP                                      | 1.0 NAA   | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple* & light green  | Compact      |
| 1.5 BAP                                      | 1.0 NAA   | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple* & light green  | Compact      |
| 2.0 BAP                                      | 1.0 NAA   | $100.00 \pm 0.00$ <sub>a</sub>                  | ++   | Purple* & light green  | Compact      |
| 0.5 BAP                                      | 0.5 2,4-D | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple & light green** | Compact      |
| 1.0 BAP                                      | 0.5 2,4-D | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple & light green** | Compact      |
| 1.5 BAP                                      | 0.5 2,4-D | $100.00 \pm 0.00$ <sub>a</sub>                  | ++   | Purple & light green*  | Compact      |
| 2.0 BAP                                      | 0.5 2,4-D | $90.00 \pm 0.06$ <sub>a</sub>                   | +  | Brown & light green*   | Compact      |
| 0.5 BAP                                      | 1.0 2,4-D | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Light green***         | Semi-compact |
| 1.0 BAP                                      | 1.0 2,4-D | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Light green***         | Semi-compact |
| 1.5 BAP                                      | 1.0 2,4-D | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Light green**          | Compact      |
| 2.0 BAP                                      | 1.0 2,4-D | $96.67 \pm 0.03$ <sub>a</sub>                   | ++   | Light green**          | Compact      |
| Gibberellin                                  | Auxin     |   |  |                        |              |
| 0.5 GA <sub>3</sub>                          | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                      | -            |
| 1.0 GA <sub>3</sub>                          | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                      | -            |
| 1.5 GA <sub>3</sub>                          | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                      | -            |
| 2.0 GA <sub>3</sub>                          | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                      | -            |

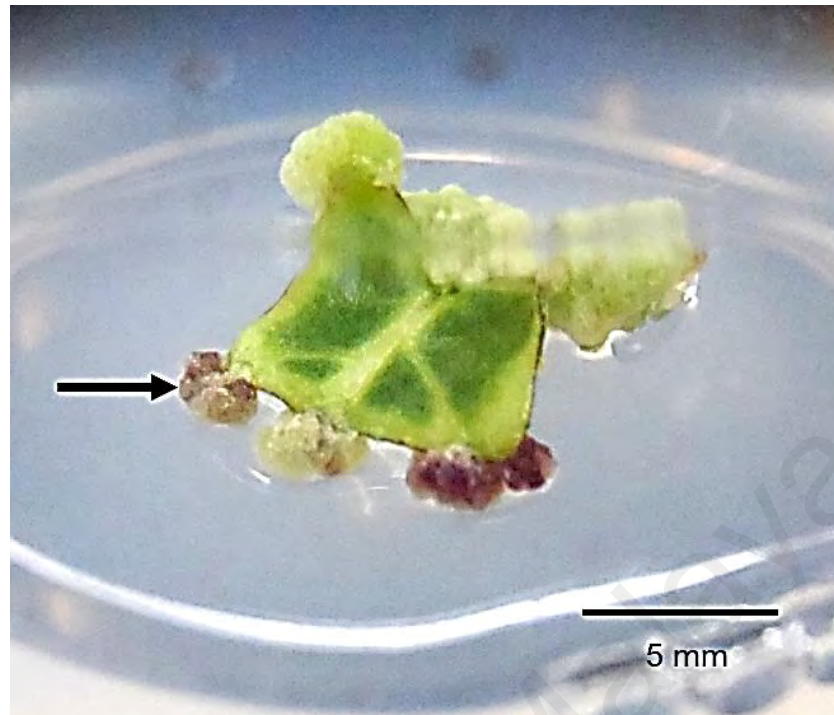
Coloured callus formation was evaluated after 6 weeks of culture with 30 explants per treatment.

<sup>x</sup> – Values represent mean (%)  $\pm$  SE of the mean followed by the same letter within columns are not significantly different at  $P = 0.05$  by DMRT.

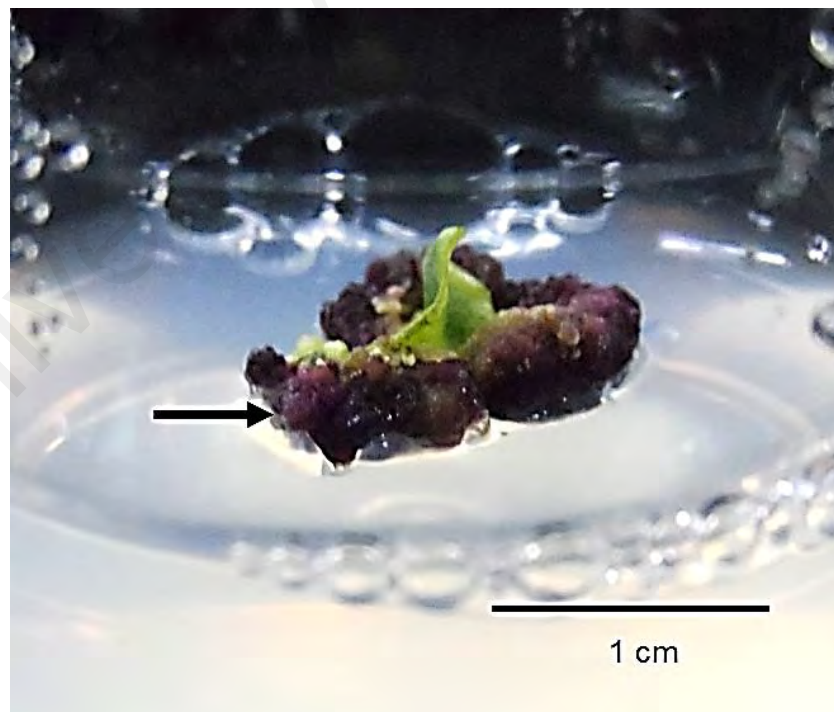
<sup>y</sup> – Observation: +++ profuse callus; ++ moderate callus; + poor callus.

<sup>z</sup> – Observation: \*\*\* extremely dominant colour; \*\* highly dominant colour; \* dominant colour.

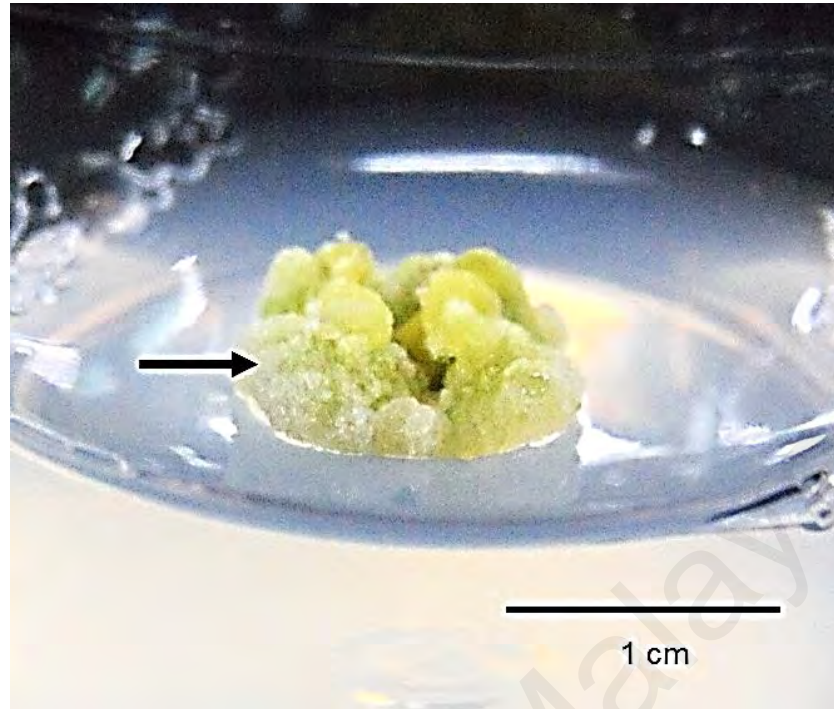




**Figure 2.2:** Leaf explant of *O. basilicum* showing callus initiation (*arrow*) after 14 days of culture on MS medium supplemented with  $0.5 \text{ mg l}^{-1}$  BAP and  $0.5 \text{ mg l}^{-1}$  NAA.



**Figure 2.3:** Formation of purple callus (*arrow*) from leaf explant of *O. basilicum* after 6 weeks of culture on MS medium supplemented with  $0.5 \text{ mg l}^{-1}$  BAP and  $0.5 \text{ mg l}^{-1}$  NAA.



**Figure 2.4:** Formation of light green callus (*arrow*) from leaf explant of *O. basilicum* after 6 weeks of culture on MS medium supplemented with  $1.0 \text{ mg l}^{-1}$  BAP and  $1.0 \text{ mg l}^{-1}$  2,4-D.

### 2.3.3 Coloured callus formation from shoot tip explants

Coloured callus formation from shoot tip explants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol were evaluated after 6 weeks of culture on the callus induction medium. For shoot tip explants, MS medium without PGR which served as control failed to induce any formation of callus. MS medium supplemented with BAP alone and MS medium supplemented with  $\text{GA}_3$  alone at all concentrations tested were also not effective in inducing callus formation from shoot tip explants. The shoot tips showed browning and became necrosis.

MS medium supplemented with different combinations of cytokinin and auxin (BAP combined with NAA and BAP combined with 2,4-D) at all concentrations tested however, were effective to induce callus formation from shoot tip explants. The calluses were observed starting to form at the base of the shoot tips and at the petiole cuttings

after 12 days of culture (Figure 2.5). Two main colours were detected in the callus formed, which were purple and light green. Of the different media tested, the best media for the highest percentage of purple callus formation from shoot tip explants were MS medium supplemented with 0.5 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> NAA and MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> NAA (Table 2.2). On these media, 100% of the explants produced callus, and the callus formed were profuse, compact, with purple callus showed high dominance over light green callus after 6 weeks of culture. On the other hand, MS medium supplemented with 0.5 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> 2,4-D and MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> 2,4-D were the best media for the highest percentage of light green callus formation from shoot tip explants (Table 2.2). On these media, 100% of the explants produced callus, and the callus formed were profuse, compact with light green callus showed extreme dominance over purple callus after 6 weeks of culture.

For BAP and NAA combination, 100% of the shoot tip explants formed callus at all concentrations tested. A low, equal balance of BAP and NAA concentration (0.5 to 1.0 mg l<sup>-1</sup> BAP combined with 0.5 mg l<sup>-1</sup> NAA) favoured the formation of profuse, purple callus from the shoot tip explants (Figure 2.6). The callus developed on the BAP and NAA-supplemented medium were compact. At low concentration of NAA (0.5 mg l<sup>-1</sup>), increasing the concentration of BAP from 1.0 to 2.0 mg l<sup>-1</sup> resulted in reduced intensity of overall callus formation. The dominance of purple callus also decreased at BAP concentration higher than 1.0 mg l<sup>-1</sup>. At all BAP concentrations tested, a slight increase of NAA concentration from 0.5 to 1.0 mg l<sup>-1</sup> enhanced the formation of light green callus but reduced the dominance of purple callus.

For BAP and 2,4-D combination, 100% of the shoot tip explants formed callus at all concentrations tested. A moderately low, equal balance of BAP and 2,4-D concentration (0.5 to 1.0 mg l<sup>-1</sup> BAP combined with 1.0 mg l<sup>-1</sup> 2,4-D) favoured the formation of profuse, light green callus from shoot tips (Figure 2.7). The callus developed on the BAP and 2,4-D-supplemented medium were semi-compact to compact. At low concentration of 2,4-D (0.5 mg l<sup>-1</sup>), increasing the concentration of BAP from 1.0 to 2.0 mg l<sup>-1</sup> resulted in reduced intensity of overall callus formation. The dominance of light green callus also decreased at BAP concentration higher than 1.0 mg l<sup>-1</sup>. At all BAP concentrations tested, a slight increase of 2,4-D concentration from 0.5 to 1.0 mg l<sup>-1</sup> enhanced the formation and dominance of light green callus but suppressed the formation of purple callus.

**Table 2.2:** Coloured callus formation from shoot tip explants of *O. basilicum* cultured on MS medium supplemented with different PGRs, maintained at  $25 \pm 2^\circ\text{C}$  under 16 hours of light and 8 hours of darkness, with an irradiance of  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

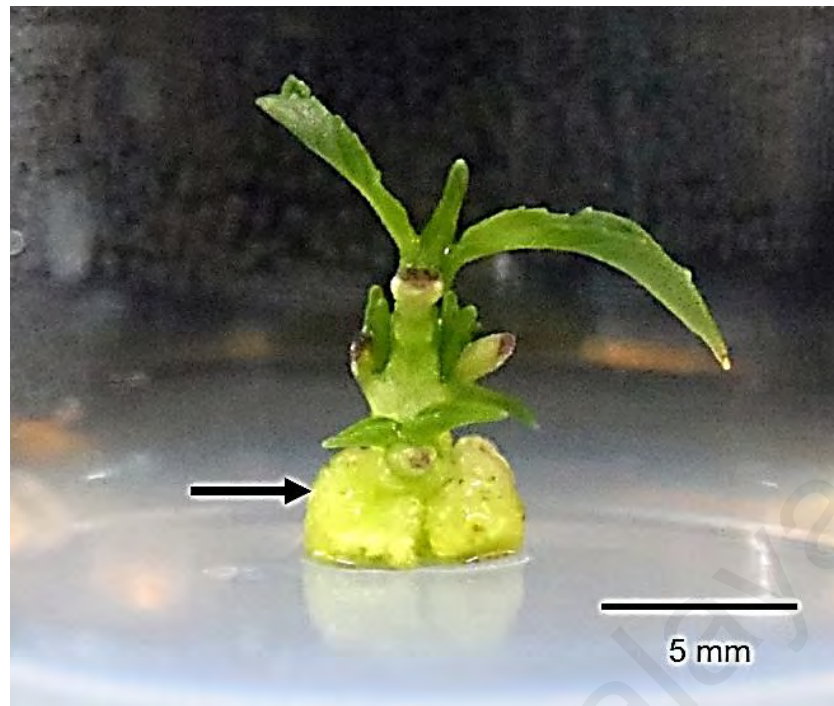
| Concentration of PGRs ( $\text{mg l}^{-1}$ ) |           | Mean callus formation (%) $\pm$ SE <sup>x</sup> | Intensity of callus formation <sup>y</sup> | Colour <sup>z</sup>     | Texture |
|--|-----------|---|--|-------------------------|---------|
| Cytokinin                                    | Auxin     |   |  |                         |         |
| 0  | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                       | -       |
| 0.5 BAP                                      | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                       | -       |
| 1.0 BAP                                      | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                       | -       |
| 1.5 BAP                                      | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                       | -       |
| 2.0 BAP                                      | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                       | -       |
| 0.5 BAP                                      | 0.5 NAA   | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple** & light green  | Compact |
| 1.0 BAP                                      | 0.5 NAA   | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple** & light green  | Compact |
| 1.5 BAP                                      | 0.5 NAA   | $100.00 \pm 0.00$ <sub>a</sub>                  | ++   | Purple* & light green   | Compact |
| 2.0 BAP                                      | 0.5 NAA   | $100.00 \pm 0.00$ <sub>a</sub>                  | ++   | Purple* & light green   | Compact |
| 0.5 BAP                                      | 1.0 NAA   | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple* & light green*  | Compact |
| 1.0 BAP                                      | 1.0 NAA   | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple* & light green   | Compact |
| 1.5 BAP                                      | 1.0 NAA   | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple* & light green   | Compact |
| 2.0 BAP                                      | 1.0 NAA   | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple* & light green   | Compact |
| 0.5 BAP                                      | 0.5 2,4-D | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple & light green**  | Compact |
| 1.0 BAP                                      | 0.5 2,4-D | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple & light green**  | Compact |
| 1.5 BAP                                      | 0.5 2,4-D | $100.00 \pm 0.00$ <sub>a</sub>                  | ++   | Purple & light green*   | Compact |
| 2.0 BAP                                      | 0.5 2,4-D | $100.00 \pm 0.00$ <sub>a</sub>                  | +  | Purple & light green*   | Compact |
| 0.5 BAP                                      | 1.0 2,4-D | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple & light green*** | Compact |
| 1.0 BAP                                      | 1.0 2,4-D | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple & light green*** | Compact |
| 1.5 BAP                                      | 1.0 2,4-D | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple & light green**  | Compact |
| 2.0 BAP                                      | 1.0 2,4-D | $100.00 \pm 0.00$ <sub>a</sub>                  | +++  | Purple & light green**  | Compact |
| Gibberellin                                  | Auxin     |   |  |                         |         |
| 0.5 GA <sub>3</sub>                          | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                       | -       |
| 1.0 GA <sub>3</sub>                          | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                       | -       |
| 1.5 GA <sub>3</sub>                          | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                       | -       |
| 2.0 GA <sub>3</sub>                          | 0         | $0.00 \pm 0.00$ <sub>b</sub>                    | -  | -                       | -       |

Coloured callus formation was evaluated after 6 weeks of culture with 30 explants per treatment.

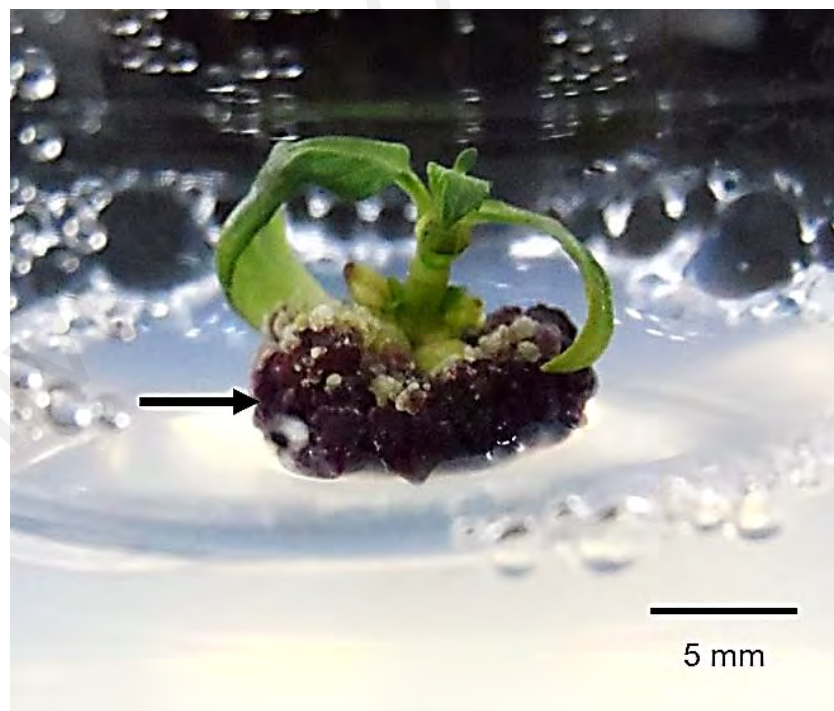
<sup>x</sup> – Values represent mean (%)  $\pm$  SE of the mean followed by the same letter within columns are not significantly different at  $P = 0.05$  by DMRT.

<sup>y</sup> – Observation: +++ profuse callus; ++ moderate callus; + poor callus.

<sup>z</sup> – Observation: \*\*\* extremely dominant colour; \*\* highly dominant colour; \* dominant colour.

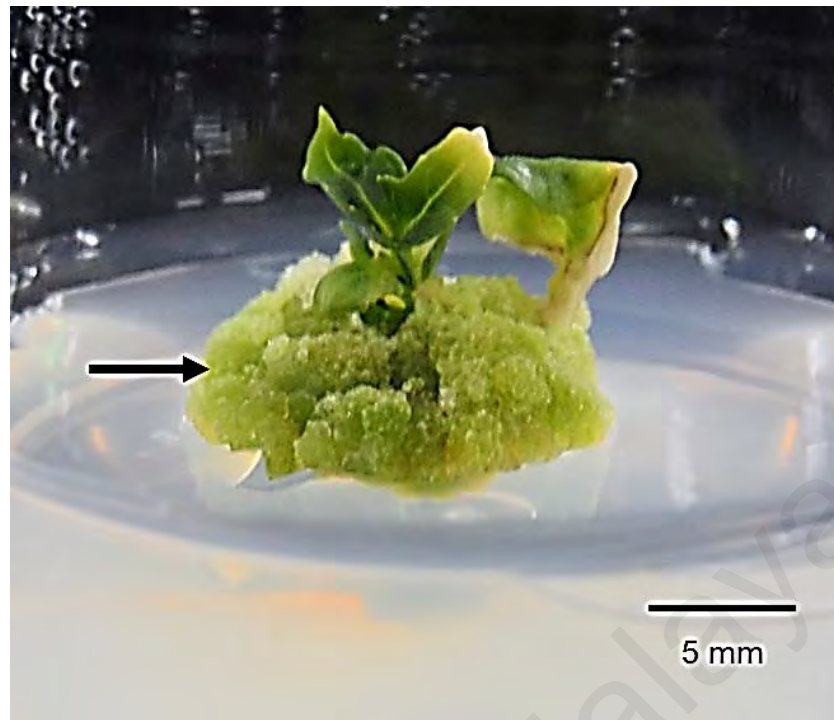


**Figure 2.5:** Shoot tip explant of *O. basilicum* showing callus initiation (*arrow*) after 12 days of culture on MS medium supplemented with  $0.5 \text{ mg l}^{-1}$  BAP and  $0.5 \text{ mg l}^{-1}$  NAA.



**Figure 2.6:** Formation of purple callus (*arrow*) from shoot tip explant of *O. basilicum* after 6 weeks of culture on MS medium supplemented with  $0.5 \text{ mg l}^{-1}$  BAP and  $0.5 \text{ mg l}^{-1}$  NAA.





**Figure 2.7:** Formation of light green callus (*arrow*) from shoot tip explant of *O. basilicum* after 6 weeks of culture on MS medium supplemented with 0.5 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> 2,4-D.

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## 2.4 Summary of Results

1. An efficient protocol for the induction of coloured callus (purple and light green) formation from leaf and shoot tip explants of *O. basilicum* 'Sweet Thai', methyl chavicol was developed.
2. MS medium supplemented with 0.5 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> NAA and MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> NAA induced the highest percentage of purple callus formation (100%) from leaf explants after 6 weeks of culture. The calluses were profuse, compact, and purple-dominant.
3. MS medium supplemented with 0.5 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> 2,4-D and MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> 2,4-D induced the highest percentage of light green callus formation (100%) from leaf explants after 6 weeks of culture. The calluses were profuse, semi-compact, and light green-dominant.
4. MS medium supplemented with 0.5 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> NAA and MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> NAA induced the highest percentage of purple callus formation (100%) from shoot tip explants after 6 weeks of culture. The calluses were profuse, compact, and purple-dominant.
5. MS medium supplemented with 0.5 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> 2,4-D and MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> 2,4-D induced the highest percentage of light green callus formation (100%) from shoot tip explants after 6 weeks of culture. The calluses were profuse, compact and light green-dominant.



## CHAPTER 3: *IN VITRO* SHOOT MULTIPLICATION AND ROOTING OF

### *Ocimum basilicum* L.

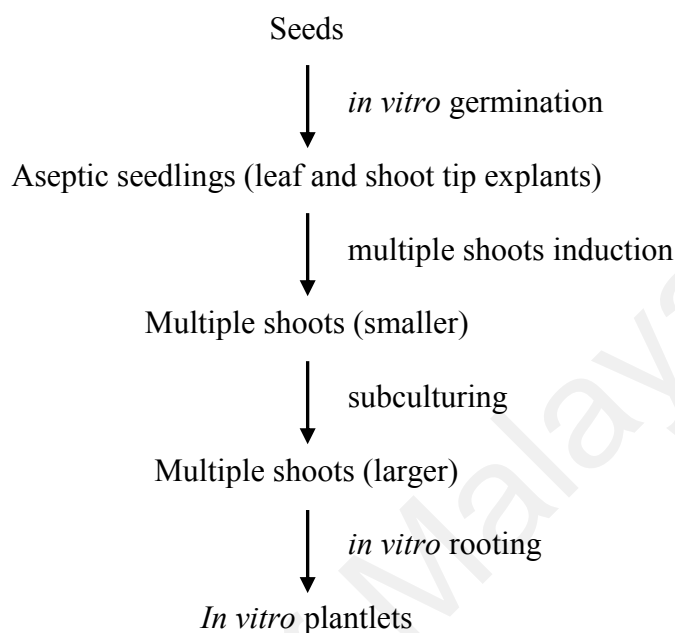
#### 3.1 Objectives of the Experiment

Micropropagation refers to the practice of rapidly multiplying stock plant material to produce a large number of genetically identical progeny plants using aseptic plant tissue culture methods. Besides nutrient medium, carbon source, and pH, the response of a plant species towards shoot proliferation is influenced by PGRs, and the requirement for PGRs varies with the type of plant materials (explants) used. The shoots grown *in vitro* need to be rooted to form new plantlets with efficient rooting system.

In this chapter, the objective of the experiment was to identify the optimum PGR or combination of PGRs that would best support induction of shoot multiplication from leaf and shoot tip explants, and to measure the efficacy of half-strength MS basal medium to promote *in vitro* rooting from multiple shoots of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol for production of complete *in vitro* plantlets. In this experiment, leaf and shoot tip explants from two-month-old aseptic seedlings of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol were cultured on MS medium with 3% sucrose, and 0.8% agar at pH 5.8; and different combinations and concentrations of PGRs were tested for maximum shoot multiplication from the explants. After that, the multiple shoots formed were subcultured on the rooting media containing half-strength MS medium, 3% sucrose, and 0.8% agar at pH 5.8.

## 3.2 Materials and Methods

### 3.2.1 Experimental outline



### 3.2.2 *In vitro* germination of seeds

Seeds of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol were surface sterilized with 70% Clorox plus Tween-20, 40% Clorox, 20% Clorox, and 70% ethanol, and then rinsed with sterile distilled water, using procedures as described in Section 2.2.3. Subsequently, the seeds were germinated on MS basal medium, consisting of 4.4 g of MS medium, 30 g of sucrose, and 8 g of agar per 1 l of medium at pH  $5.8 \pm 0.1$  in glass jars. All seed cultures were incubated in a growth chamber maintained at  $25 \pm 2^\circ\text{C}$  under 16 hours of light and 8 hours of darkness, with an irradiance of  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  provided by Philips Lifemax TL-D 18W cool daylight fluorescent tubes.

### 3.2.3 Induction of multiple shoots formation

Leaf explants (approximately  $5 \times 5 \text{ mm}^2$  area) and shoot tip explants (approximately 7 mm length) from two-month-old aseptic seedlings were cultured on

shoot induction media (1 l of medium consisted of 4.4 g of MS medium, 30 g of sucrose, and 8 g of agar at pH  $5.8 \pm 0.1$ , added with different combinations of PGRs), using procedures as described in Section 2.2.4 to induce multiple shoots formation. PGR-free MS medium was used as a control. The PGR combinations and concentrations tested were as listed below:

- (1) MS basal medium (no PGR)
- (2) MS medium + 0.5 mg l<sup>-1</sup> BAP
- (3) MS medium + 1.0 mg l<sup>-1</sup> BAP
- (4) MS medium + 1.5 mg l<sup>-1</sup> BAP
- (5) MS medium + 2.0 mg l<sup>-1</sup> BAP
- (6) MS medium + 0.5 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> NAA
- (7) MS medium + 1.0 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> NAA
- (8) MS medium + 1.5 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> NAA
- (9) MS medium + 2.0 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> NAA
- (10) MS medium + 0.5 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> NAA
- (11) MS medium + 1.0 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> NAA
- (12) MS medium + 1.5 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> NAA
- (13) MS medium + 2.0 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> NAA
- (14) MS medium + 0.5 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> 2,4-D
- (15) MS medium + 1.0 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> 2,4-D
- (16) MS medium + 1.5 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> 2,4-D
- (17) MS medium + 2.0 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> 2,4-D
- (18) MS medium + 0.5 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> 2,4-D
- (19) MS medium + 1.0 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> 2,4-D
- (20) MS medium + 1.5 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> 2,4-D
- (21) MS medium + 2.0 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> 2,4-D

- (22) MS medium + 0.5 mg l<sup>-1</sup> GA<sub>3</sub>
- (23) MS medium + 1.0 mg l<sup>-1</sup> GA<sub>3</sub>
- (24) MS medium + 1.5 mg l<sup>-1</sup> GA<sub>3</sub>
- (25) MS medium + 2.0 mg l<sup>-1</sup> GA<sub>3</sub>

All the shoot tip and leaf cultures were incubated in a growth chamber maintained at 25 ± 2°C under 16 hours of light and 8 hours of darkness, with an irradiance of 50 μmol m<sup>-2</sup> s<sup>-1</sup> provided by Philips Lifemax TL-D 18W cool daylight fluorescent tubes. The percentage of explants showing shoot multiplication, mean number of shoots per explant, and mean shoot lengths were evaluated after 8 weeks of culture. Thirty explants were used per treatment.

#### **3.2.4 Subculturing**

Multiple shoots were subcultured to fresh medium at 2 months interval to provide enough nutrient supply for good plant growth.

#### **3.2.5 *In vitro* rooting**

Multiple shoots, 3.5 to 4.0 cm in length and with four or more leaves, were excised individually and transferred from the shoot induction medium to the rooting medium containing approximately 45 ml of half-strength MS basal medium (1 l of medium consisted of 2.2 g of MS medium, 30 g of sucrose, and 8 g of agar at pH 5.8 ± 0.1) in 300-ml glass jars. All cultures were incubated in a growth chamber maintained at 25 ± 2°C under 16 hours of light and 8 hours of darkness, with an irradiance of 50 μmol m<sup>-2</sup> s<sup>-1</sup> provided by Philips Lifemax TL-D 18W cool daylight fluorescent tubes. The percentage of shoots showing root formation and mean root lengths were evaluated after 4 weeks of rooting using thirty shoots.

### **3.2.6 Statistical analysis**

Data for multiple shoots induction (Section 3.2.3) was statistically analysed using one-way analysis of variance (ANOVA) with confidence intervals calculated via Duncan's multiple range test (DMRT) at  $P = 0.05$ , using IBM SPSS statistics version 22 software. Data for rooting (Section 3.2.5) was calculated via descriptive statistics, using IBM SPSS statistics version 22 software. Results were presented as mean  $\pm$  standard error (SE) of the mean.

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### 3.3 Results

#### 3.3.1 *In vitro* shoot multiplication from leaf explants

*In vitro* shoot multiplication induced from leaf explants of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol was evaluated after 8 weeks of culture on shoot induction medium. When cultured on MS medium supplemented with and without PGRs, the leaf explants failed to form any shoots in all the treatments tested. The leaves either produced callus or became necrosis, depending on the PGR combinations and concentrations used as explained in Chapter 2, Section 2.3.2.

#### 3.3.2 *In vitro* shoot multiplication from shoot tip explants

*In vitro* shoot multiplication induced from shoot tip explants of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol was evaluated after 8 weeks of culture on the shoot induction medium. The mean number of shoots formed per explant under the influence of different PGRs was shown in Figure 3.1. Shoot tip explants cultured on PGR-free MS medium regenerated complete plantlets with no axillary shoot formation, even after 8 weeks of culture. A mean of  $1.30 \pm 0.11$  shoot per explant was formed on this medium. The MS medium without PGR unable to induce shoot multiplication from the shoot tip explants.

On MS medium supplemented with different PGRs (BAP alone, BAP combined with NAA, and GA<sub>3</sub> alone), the shoot tip explants showed initial bud break within 10 to 12 days of culture (Figure 3.2) and later shoots formed from the axillary buds and apical buds (Figure 3.3). The number of shoots formed averaged between two to five shoots per explant, depending on the combination and concentration of PGRs used. Of the different media tested, MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP was found to be

the best for shoot formation, producing 100% shoot multiplication with a mean of  $5.00 \pm 0.28$  shoots per explant averaging  $1.42 \pm 0.11$  cm in length after 8 weeks of culture (Table 3.1).

MS medium supplemented with BAP alone at all concentrations tested produced 100% shoot multiplication. Increasing the BAP concentration from 0.5 to 1.0 mg l<sup>-1</sup> enhanced the number of shoots per explant, producing a mean of  $3.60 \pm 0.17$  and  $5.00 \pm 0.28$  shoots per explant, respectively. Further increase in BAP concentration reduced the number of shoots per explant (1.5 mg l<sup>-1</sup> BAP produced a mean of  $4.50 \pm 0.29$  shoots per explant; 2.0 mg l<sup>-1</sup> BAP produced a mean of  $4.63 \pm 0.19$  shoots per explant) and resulted in stunted growth of shoots, but the differences were not significant.

For MS medium supplemented with BAP and NAA combination, at low concentration of NAA (0.5 to 1.0 mg l<sup>-1</sup>), increasing the concentration of BAP from 0.5 to 2.0 mg l<sup>-1</sup> enhanced the multiplication percentage and number of shoots per explant (0.5 mg l<sup>-1</sup> NAA combined with 0.5, 1.0, 1.5 and 2.0 mg l<sup>-1</sup> BAP produced a mean of  $2.33 \pm 0.19$ ,  $2.60 \pm 0.18$ ,  $3.20 \pm 0.30$  and  $4.07 \pm 0.27$  shoots per explant, respectively; 1.0 mg l<sup>-1</sup> NAA combined with 0.5, 1.0, 1.5 and 2.0 mg l<sup>-1</sup> BAP produced a mean of  $2.29 \pm 0.29$ ,  $2.40 \pm 0.25$ ,  $2.63 \pm 0.21$  and  $2.67 \pm 0.21$  shoots per explant, respectively). BAP combined with NAA produced a lower mean number of shoots per explant compared to BAP alone, as their combination induced callusing. For all BAP concentrations tested, when the concentration of NAA was increased from 0.5 to 1.0 mg l<sup>-1</sup>, the number of shoots per explant decreased. Increasing the concentration of BAP to NAA from 0.5 to 2.0 mg l<sup>-1</sup> minimized the reduction in the number of shoots through suppressed callusing, thus enhancing the multiplication percentage and number of shoots per explant.

MS medium supplemented with combination of BAP and 2,4-D at all concentrations tested resulted in no shoot multiplication. The growth of shoot tips remained stagnant with no development, but they produced callus at the base.

Gibberellic acid ( $GA_3$ ), applied singly, especially at  $1.5 \text{ mg l}^{-1}$  stimulated shoot elongation via increased mean shoot length up to  $2.73 \pm 0.24 \text{ cm}$  after 8 weeks of culture. However, its effect on shoot proliferation was small.  $GA_3$  at concentrations between  $0.5$  and  $2.0 \text{ mg l}^{-1}$  produced an average of only two shoots per explant.

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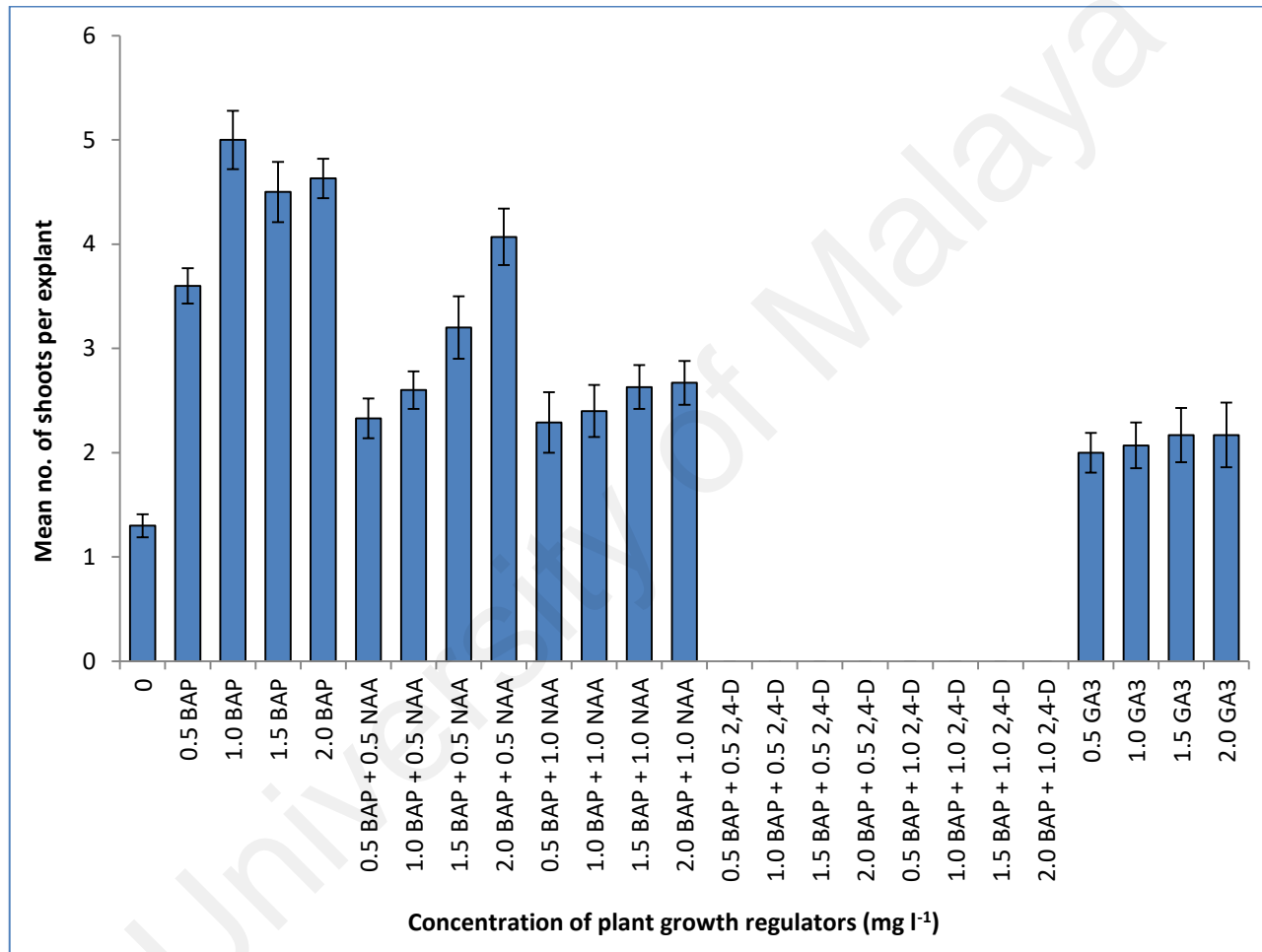


**Table 3.1:** Multiple shoots formation from shoot tip explants of *O. basilicum* cultured on MS medium supplemented with different PGRs, maintained at  $25 \pm 2^\circ\text{C}$  under 16 hours of light and 8 hours of darkness, with an irradiance of  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

| Concentration of PGRs<br>( $\text{mg l}^{-1}$ ) |           | Mean shoot<br>multiplication<br>(%) $\pm$ SE <sup>x</sup> | Mean number of<br>shoots per explant<br>$\pm$ SE <sup>y</sup> | Mean shoot<br>length (cm) $\pm$<br>SE <sup>z</sup> |
|---|-----------|---|---|--|
| Cytokinin                                       | Auxin     |   |   |  |
| 0   | 0         | 23.33 $\pm$ 0.08 <sub>e</sub>                             | 1.30 $\pm$ 0.11 <sub>g</sub>                                  | 1.83 $\pm$ 0.12 <sub>b</sub>                       |
| 0.5 BAP   | 0         | 100.00 $\pm$ 0.00 <sub>a</sub>                            | 3.60 $\pm$ 0.17 <sub>cd</sub>                                 | 1.26 $\pm$ 0.08 <sub>cd</sub>                      |
| 1.0 BAP   | 0         | 100.00 $\pm$ 0.00 <sub>a</sub>                            | 5.00 $\pm$ 0.28 <sub>a</sub>                                  | 1.42 $\pm$ 0.11 <sub>c</sub>                       |
| 1.5 BAP   | 0         | 100.00 $\pm$ 0.00 <sub>a</sub>                            | 4.50 $\pm$ 0.29 <sub>ab</sub>                                 | 1.41 $\pm$ 0.07 <sub>cd</sub>                      |
| 2.0 BAP   | 0         | 100.00 $\pm$ 0.00 <sub>a</sub>                            | 4.63 $\pm$ 0.19 <sub>ab</sub>                                 | 1.19 $\pm$ 0.07 <sub>cd</sub>                      |
| 0.5 BAP   | 0.5 NAA   | 76.67 $\pm$ 0.08 <sub>bc</sub>                            | 2.33 $\pm$ 0.19 <sub>f</sub>                                  | 1.39 $\pm$ 0.13 <sub>cd</sub>                      |
| 1.0 BAP   | 0.5 NAA   | 83.33 $\pm$ 0.07 <sub>ab</sub>                            | 2.60 $\pm$ 0.18 <sub>ef</sub>                                 | 1.17 $\pm$ 0.10 <sub>cd</sub>                      |
| 1.5 BAP   | 0.5 NAA   | 93.33 $\pm$ 0.05 <sub>ab</sub>                            | 3.20 $\pm$ 0.30 <sub>de</sub>                                 | 1.20 $\pm$ 0.09 <sub>cd</sub>                      |
| 2.0 BAP   | 0.5 NAA   | 96.67 $\pm$ 0.03 <sub>ab</sub>                            | 4.07 $\pm$ 0.27 <sub>bc</sub>                                 | 1.14 $\pm$ 0.10 <sub>cd</sub>                      |
| 0.5 BAP   | 1.0 NAA   | 60.00 $\pm$ 0.09 <sub>cd</sub>                            | 2.29 $\pm$ 0.29 <sub>f</sub>                                  | 0.69 $\pm$ 0.06 <sub>d</sub>                       |
| 1.0 BAP   | 1.0 NAA   | 60.00 $\pm$ 0.09 <sub>cd</sub>                            | 2.40 $\pm$ 0.25 <sub>f</sub>                                  | 0.97 $\pm$ 0.12 <sub>d</sub>                       |
| 1.5 BAP   | 1.0 NAA   | 83.33 $\pm$ 0.07 <sub>ab</sub>                            | 2.63 $\pm$ 0.21 <sub>ef</sub>                                 | 1.21 $\pm$ 0.10 <sub>cd</sub>                      |
| 2.0 BAP   | 1.0 NAA   | 86.67 $\pm$ 0.06 <sub>ab</sub>                            | 2.67 $\pm$ 0.21 <sub>ef</sub>                                 | 1.31 $\pm$ 0.12 <sub>cd</sub>                      |
| 0.5 BAP   | 0.5 2,4-D | 0.00 $\pm$ 0.00 <sub>f</sub>                              | 0.00 $\pm$ 0.00 <sub>h</sub>                                  | 0.00 $\pm$ 0.00 <sub>e</sub>                       |
| 1.0 BAP   | 0.5 2,4-D | 0.00 $\pm$ 0.00 <sub>f</sub>                              | 0.00 $\pm$ 0.00 <sub>h</sub>                                  | 0.00 $\pm$ 0.00 <sub>e</sub>                       |
| 1.5 BAP   | 0.5 2,4-D | 0.00 $\pm$ 0.00 <sub>f</sub>                              | 0.00 $\pm$ 0.00 <sub>h</sub>                                  | 0.00 $\pm$ 0.00 <sub>e</sub>                       |
| 2.0 BAP   | 0.5 2,4-D | 0.00 $\pm$ 0.00 <sub>f</sub>                              | 0.00 $\pm$ 0.00 <sub>h</sub>                                  | 0.00 $\pm$ 0.00 <sub>e</sub>                       |
| 0.5 BAP   | 1.0 2,4-D | 0.00 $\pm$ 0.00 <sub>f</sub>                              | 0.00 $\pm$ 0.00 <sub>h</sub>                                  | 0.00 $\pm$ 0.00 <sub>e</sub>                       |
| 1.0 BAP   | 1.0 2,4-D | 0.00 $\pm$ 0.00 <sub>f</sub>                              | 0.00 $\pm$ 0.00 <sub>h</sub>                                  | 0.00 $\pm$ 0.00 <sub>e</sub>                       |
| 1.5 BAP   | 1.0 2,4-D | 0.00 $\pm$ 0.00 <sub>f</sub>                              | 0.00 $\pm$ 0.00 <sub>h</sub>                                  | 0.00 $\pm$ 0.00 <sub>e</sub>                       |
| 2.0 BAP   | 1.0 2,4-D | 0.00 $\pm$ 0.00 <sub>f</sub>                              | 0.00 $\pm$ 0.00 <sub>h</sub>                                  | 0.00 $\pm$ 0.00 <sub>e</sub>                       |
| Gibberellin                                     | Auxin     |   |   |  |
| 0.5 GA <sub>3</sub>                             | 0         | 60.00 $\pm$ 0.09 <sub>cd</sub>                            | 2.00 $\pm$ 0.19 <sub>f</sub>                                  | 1.80 $\pm$ 0.15 <sub>b</sub>                       |
| 1.0 GA <sub>3</sub>                             | 0         | 60.00 $\pm$ 0.09 <sub>cd</sub>                            | 2.07 $\pm$ 0.22 <sub>f</sub>                                  | 2.16 $\pm$ 0.20 <sub>b</sub>                       |
| 1.5 GA <sub>3</sub>                             | 0         | 50.00 $\pm$ 0.09 <sub>d</sub>                             | 2.17 $\pm$ 0.26 <sub>f</sub>                                  | 2.73 $\pm$ 0.24 <sub>a</sub>                       |
| 2.0 GA <sub>3</sub>                             | 0         | 50.00 $\pm$ 0.09 <sub>d</sub>                             | 2.17 $\pm$ 0.31 <sub>f</sub>                                  | 2.16 $\pm$ 0.20 <sub>b</sub>                       |

Shoot proliferation was evaluated after 8 weeks of culture with 30 explants per treatment.

<sup>x</sup>, <sup>y</sup>, <sup>z</sup> – Values represent mean  $\pm$  standard error of the mean followed by the same letter within columns are not significantly different at  $P = 0.05$  by DMRT.



**Figure 3.1:** Effect of concentration of PGRs on mean number of shoots per explant from shoot tip explants of *O. basilicum* cultured on MS medium. Error bars are calculated as SE of the mean.



**Figure 3.2:** Shoot tip explant of *O. basilicum* showing axillary bud formation (*arrow*) after 12 days of culture on MS medium supplemented with  $1.0 \text{ mg l}^{-1}$  BAP.



**Figure 3.3:** Formation of multiple shoots (*arrow*) from shoot tip explant of *O. basilicum* after 8 weeks of culture on MS medium supplemented with  $1.0 \text{ mg l}^{-1}$  BAP.

### 3.3.3 *In vitro* rooting

Multiple shoots of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol were rooted on rooting medium containing half-strength MS medium without plant growth regulator. Individual multiple shoots showed 100% rooting with mean root length of  $4.21 \pm 0.29$  cm after 4 weeks of culture on the rooting medium (Table 3.2; Figure 3.4). The plantlets had good rooting system for transplantation after 6 weeks of rooting (Figure 3.5).

**Table 3.2:** *In vitro* rooting of *O. basilicum*.

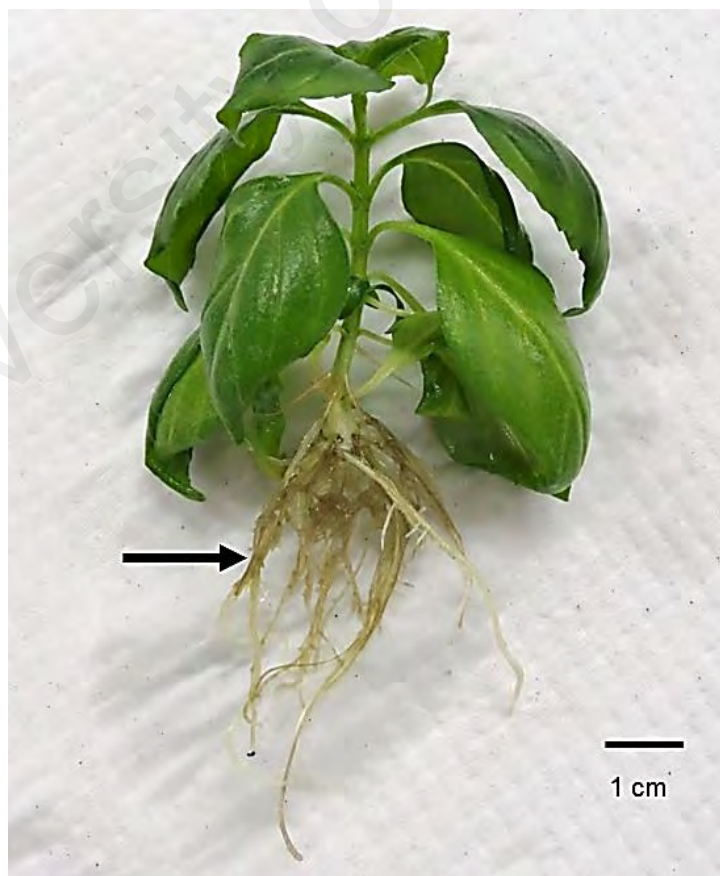
| Treatment  | Results  |
|--|--|
| <i>In vitro</i> rooting on half-strength MS basal medium | <ul style="list-style-type: none"><li>• Percentage of rooting plantlets <math>\pm</math> SE<sup>z</sup>:<br/>100.00% <math>\pm</math> 0.00.</li><li>• Mean root length <math>\pm</math> SE<sup>z</sup>:<br/>4.21 <math>\pm</math> 0.29 cm.</li></ul> |

*In vitro* rooting was evaluated after 4 weeks of culture with 30 shoots per treatment.

<sup>z</sup> – Values represent mean  $\pm$  SE of the mean by descriptive statistics.



**Figure 3.4:** Formation of roots (*arrow*) from multiple shoots of *O. basilicum* after 4 weeks of rooting on half-strength MS basal medium.



**Figure 3.5:** Roots (*arrow*) of *in vitro* plantlet of *O. basilicum* after 6 weeks of rooting on half-strength MS basal medium.

### 3.4 Summary of Results

1. An efficient protocol for the induction of shoot multiplication from shoot tip explants of *O. basilicum* 'Sweet Thai', methyl chavicol was developed for mass production of multiple shoots.
2. MS medium with and without supplementation of PGRs at all concentrations tested did not induce shoot formation from leaf explants.
3. MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP induced the highest percentage of shoot multiplication from shoot tip explants, with 100% of explants producing multiple shoots, having a mean of 5.00 ± 0.28 shoots per explant averaging 1.42 ± 0.11 cm in length after 8 weeks of culture.
4. All (100%) multiple shoots showed root formation with mean root length of 4.21 ± 0.29 cm after 4 weeks of *in vitro* rooting on half-strength MS basal medium.

## CHAPTER 4: *IN VITRO* FLOWERING OF *Ocimum basilicum* L.

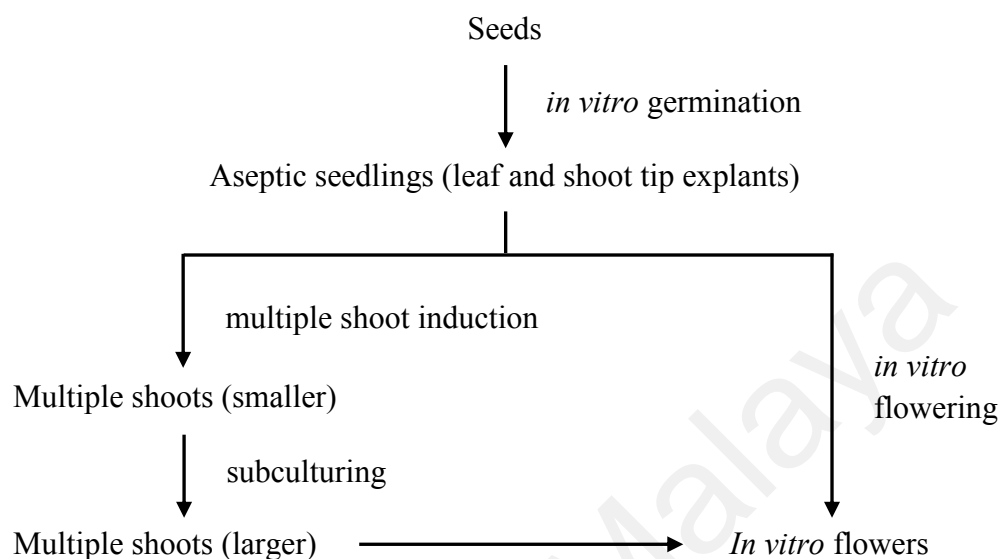
### 4.1 Objectives of the Experiment

Flower is the reproductive organ of an angiosperm. The aesthetic structure, colour, and fragrance of a flower serve an essential purpose to attract pollinators to facilitate fertilization. Some flowers contain pigments and oils which are biologically active. *In vitro* flowering can be useful for the study of flowering process, for breeding programs and for the production of specific compounds from floral organs. There is considerable variability in the requirements of PGRs, temperature, light regime and nutritional factors for *in vitro* flower development in explants from different species.

In this chapter, the objective of the experiment was to identify the optimum PGR or combination of PGRs that would best support induction of *in vitro* flowering from leaf and shoot tip explants, and to measure its efficacy to induce *in vitro* flowering from multiple shoots of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol for production of normal flowers. In this experiment, leaf and shoot tip explants from two-month-old aseptic seedlings of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol were cultured on culture media containing MS medium, 3% sucrose, and 0.8% agar at pH 5.8; and different combinations and concentrations of PGRs were tested for maximum *in vitro* flowering from the explants. After that, the best flower induction medium was tested for its efficacy to induce *in vitro* flowering from multiple shoots obtained in Chapter 3.

## 4.2 Materials and Methods

### 4.2.1 Experimental outline



### 4.2.2 *In vitro* germination of seeds

Seeds of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol were surface sterilized with 70% Clorox plus Tween-20, 40% Clorox, 20% Clorox, and 70% ethanol, and then rinsed with sterile distilled water, using procedures as described in Section 2.2.3. Next, the seeds were germinated on MS basal medium, consisting of 4.4 g of MS medium, 30 g of sucrose, and 8 g of agar per 1 l of medium at pH  $5.8 \pm 0.1$  in glass jars. All seed cultures were incubated in a growth chamber maintained at  $25 \pm 2^\circ\text{C}$  under 16 hours of light and 8 hours of darkness, with an irradiance of  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  provided by Philips Lifemax TL-D 18W cool daylight fluorescent tubes.

### 4.2.3 Induction of *in vitro* flowering

Two sets of experiments were carried out in the induction of *in vitro* flowering experiment. In Setup I, *in vitro* flowering was induced from leaf and shoot tip explants of two-month-old aseptic seedlings. Leaf explants (approximately  $5 \times 5 \text{ mm}^2$  area) and



shoot tip explants (approximately 7 mm length) from two-month-old aseptic seedlings were cultured on flower induction media (1 l of medium consisted of 4.4 g of MS medium, 30 g of sucrose, and 8 g of agar at pH  $5.8 \pm 0.1$ , added with different combinations of PGRs), using procedures as described in Section 2.2.4. PGR-free MS medium was used as a control. The PGR combinations and concentrations tested were as listed below:

- (1) MS basal medium (no PGR)
- (2) MS medium + 0.5 mg l<sup>-1</sup> BAP
- (3) MS medium + 1.0 mg l<sup>-1</sup> BAP
- (4) MS medium + 1.5 mg l<sup>-1</sup> BAP
- (5) MS medium + 2.0 mg l<sup>-1</sup> BAP
- (6) MS medium + 0.5 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> NAA
- (7) MS medium + 1.0 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> NAA
- (8) MS medium + 1.5 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> NAA
- (9) MS medium + 2.0 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> NAA
- (10) MS medium + 0.5 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> NAA
- (11) MS medium + 1.0 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> NAA
- (12) MS medium + 1.5 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> NAA
- (13) MS medium + 2.0 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> NAA
- (14) MS medium + 0.5 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> 2,4-D
- (15) MS medium + 1.0 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> 2,4-D
- (16) MS medium + 1.5 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> 2,4-D
- (17) MS medium + 2.0 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> 2,4-D
- (18) MS medium + 0.5 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> 2,4-D
- (19) MS medium + 1.0 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> 2,4-D
- (20) MS medium + 1.5 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> 2,4-D

- (21) MS medium + 2.0 mg l<sup>-1</sup> BAP + 1.0 mg l<sup>-1</sup> 2,4-D
- (22) MS medium + 0.5 mg l<sup>-1</sup> GA<sub>3</sub>
- (23) MS medium + 1.0 mg l<sup>-1</sup> GA<sub>3</sub>
- (24) MS medium + 1.5 mg l<sup>-1</sup> GA<sub>3</sub>
- (25) MS medium + 2.0 mg l<sup>-1</sup> GA<sub>3</sub>

All the shoot tip and leaf cultures were incubated in a growth chamber maintained at 25 ± 2 °C under 16 hours of light and 8 hours of darkness, with an irradiance of 50 μmol m<sup>-2</sup> s<sup>-1</sup> provided by Philips Lifemax TL-D 18W cool daylight fluorescent tubes. The percentage of explants producing *in vitro* flowers was evaluated until the 20<sup>th</sup> week of culture. Thirty explants were used per treatment. The morphology of the *in vitro* flowers produced was compared with the normal *in vivo* flowers.

In Setup II, the experiment was carried out to test the effectiveness of the PGR that induced the maximum *in vitro* flowering from leaf or shoot tip explants (in Setup I) to induce *in vitro* flowering from multiple shoots. Shoots approximately 3.0 cm in length were excised individually from five-month-old multiple shoots developed on the optimum shoot induction medium (Chapter 3) and transferred to the optimum flower induction medium in jam jars (In this experiment, multiple shoots of MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP were transferred to MS medium supplemented with 1.0 mg l<sup>-1</sup> GA<sub>3</sub>). All cultures were incubated in a growth chamber maintained at 25 ± 2°C under 16 hours of light and 8 hours of darkness, with an irradiance of 50 μmol m<sup>-2</sup> s<sup>-1</sup> provided by Philips Lifemax TL-D 18W cool daylight fluorescent tubes. The percentage of shoots with *in vitro* flowers and mean number of flowers per shoot were evaluated until the 16<sup>th</sup> week of culture. Thirty shoots were used per treatment. The

morphology of the *in vitro* flowers produced was compared with the normal *in vivo* flowers, obtained from the plant growing in a village garden.

#### **4.2.4 Statistical analysis**

Data was statistically analysed using one-way analysis of variance (ANOVA) with confidence intervals calculated via Duncan's multiple range test (DMRT) at  $P = 0.05$ , using IBM SPSS statistics version 22 software. Results were presented as mean  $\pm$  standard error (SE) of the mean.

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## 4.3 Results

### 4.3.1 *In vitro* flowering from leaf explants

*In vitro* flowering induced from leaf explants of *O. basilicum* 'Sweet' Thai, chemotype methyl chavicol was evaluated until the 20<sup>th</sup> week of culture on the flower induction media. When cultured on MS medium supplemented with and without PGRs, the leaf explants failed to form any flowers in all the treatments tested. The leaves either produced callus or became necrosis, depending on the PGR combinations and concentrations used as explained in Chapter 2, Section 2.3.2.

### 4.3.2 *In vitro* flowering from shoot tip explants

In Setup I, *in vitro* flowering was induced from shoot tip explants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol. The percentage of *in vitro* flowering under the influence of different PGRs was shown in Figure 4.1. Flower buds were initiated, and later full bloom flowers formed from the developing shoots on the flower induction medium (Figure 4.2). Of the different media tested, the highest percentage of *in vitro* flowering occurred in MS medium supplemented with 1.0 mg l<sup>-1</sup> GA<sub>3</sub>. On this medium, 40% of explants produced normal flowers *in vitro* before the 20<sup>th</sup> week of culture (Table 4.1). Normal flowers bloomed starting from the 6<sup>th</sup> week of culture on the flower induction medium (Figure 4.3). The flowers were white tinged with purple, and they were carried in spikes with six flowers per whorl. The *in vitro* flowers exhibited similar morphology compared to the normal *in vivo* flowers – both of them had corolla of 7 to 10 mm in length which comprised of a lower lip, a four-lobed upper lip, one pistil, and four stamens (Figure 4.4). A single flower lasted for about 1 to 2 weeks under *in vitro* conditions.

Shoot tip explants cultured on MS medium supplemented with GA<sub>3</sub> alone developed *in vitro* flowers at all concentrations tested. GA<sub>3</sub>, at 1.0 mg l<sup>-1</sup> induced the highest percentage of *in vitro* flowering from the shoot tip explants, producing 40% explants with normal flowers before the 20<sup>th</sup> week of culture. At GA<sub>3</sub> concentrations higher than 1.0 mg l<sup>-1</sup>, the percentage of normal *in vitro* flowering decreased. However, when treated with GA<sub>3</sub>, 10% to 13% of the flowers were abnormal and premature, emerging as early as the 2<sup>nd</sup> week of culture. The flowers appeared to be smaller than the normal flowers, lacked stamens and pistils, and sometimes lacked purple pigmentation (Figure 4.5). These abnormalities were not significant and temporary as the plants later produced normal flowers from the 6<sup>th</sup> through the 20<sup>th</sup> week.

Shoot tip explants cultured on PGR-free MS medium also developed *in vitro* flowers spontaneously, but the percentage of explants with normal flowers in this medium, which was 20%, was lower than the GA<sub>3</sub>-supplemented MS medium.

BAP either alone or in combination with NAA or 2,4-D, at all concentrations tested was ineffective for inducing *in vitro* flowering from the shoot tip explants.

**Table 4.1:** *In vitro* flowering from shoot tip explants of *O. basilicum* cultured on MS medium supplemented with different PGRs, maintained at  $25 \pm 2^\circ\text{C}$  under 16 hours of light and 8 hours of darkness, with an irradiance of  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

| <b>Setup I: <i>In vitro</i> flowering induced from shoot tip explants</b> |           |  |  |
|---|-----------|--|--|
| <b>Concentration of PGRs (mg l<sup>-1</sup>)</b>                          |           | <b>Mean normal <i>in vitro</i> flowering (%) <math>\pm</math> SE<sup>y</sup></b> | <b>Mean abnormal <i>in vitro</i> flowering (%) <math>\pm</math> SE<sup>z</sup></b> |
| Cytokinin   | Auxin     |  |  |
| 0   | 0         | 20.00 $\pm$ 0.07 <sub>bc</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 0.5 BAP   | 0         | 3.33 $\pm$ 0.03 <sub>cd</sub>  | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 1.0 BAP   | 0         | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 1.5 BAP   | 0         | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 2.0 BAP   | 0         | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 0.5 BAP   | 0.5 NAA   | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 1.0 BAP   | 0.5 NAA   | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 1.5 BAP   | 0.5 NAA   | 3.33 $\pm$ 0.03 <sub>cd</sub>  | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 2.0 BAP   | 0.5 NAA   | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 0.5 BAP   | 1.0 NAA   | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 1.0 BAP   | 1.0 NAA   | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 1.5 BAP   | 1.0 NAA   | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 2.0 BAP   | 1.0 NAA   | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 0.5 BAP   | 0.5 2,4-D | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 1.0 BAP   | 0.5 2,4-D | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 1.5 BAP   | 0.5 2,4-D | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 2.0 BAP   | 0.5 2,4-D | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 0.5 BAP   | 1.0 2,4-D | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 1.0 BAP   | 1.0 2,4-D | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 1.5 BAP   | 1.0 2,4-D | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| 2.0 BAP   | 1.0 2,4-D | 0.00 $\pm$ 0.00 <sub>d</sub>   | 0.00 $\pm$ 0.00 <sub>a</sub>   |
| Gibberellin   | Auxin     |  |  |
| 0.5 GA <sub>3</sub>   | 0         | 23.33 $\pm$ 0.08 <sub>ab</sub>   | 13.33 $\pm$ 0.06 <sub>a</sub>  |
| 1.0 GA <sub>3</sub>   | 0         | 40.00 $\pm$ 0.09 <sub>a</sub>  | 10.00 $\pm$ 0.06 <sub>a</sub>  |
| 1.5 GA <sub>3</sub>   | 0         | 23.33 $\pm$ 0.08 <sub>ab</sub>   | 10.00 $\pm$ 0.06 <sub>a</sub>  |
| 2.0 GA <sub>3</sub>   | 0         | 20.00 $\pm$ 0.07 <sub>bc</sub>   | 10.00 $\pm$ 0.06 <sub>a</sub>  |

*In vitro* flowering was evaluated until the 20<sup>th</sup> week of culture with 30 explants per treatment.

<sup>y, z</sup> – Values represent mean (%)  $\pm$  SE of the mean followed by the same letter within columns are not significantly different at  $P = 0.05$  by DMRT.

### 4.3.3 *In vitro* flowering from multiple shoots

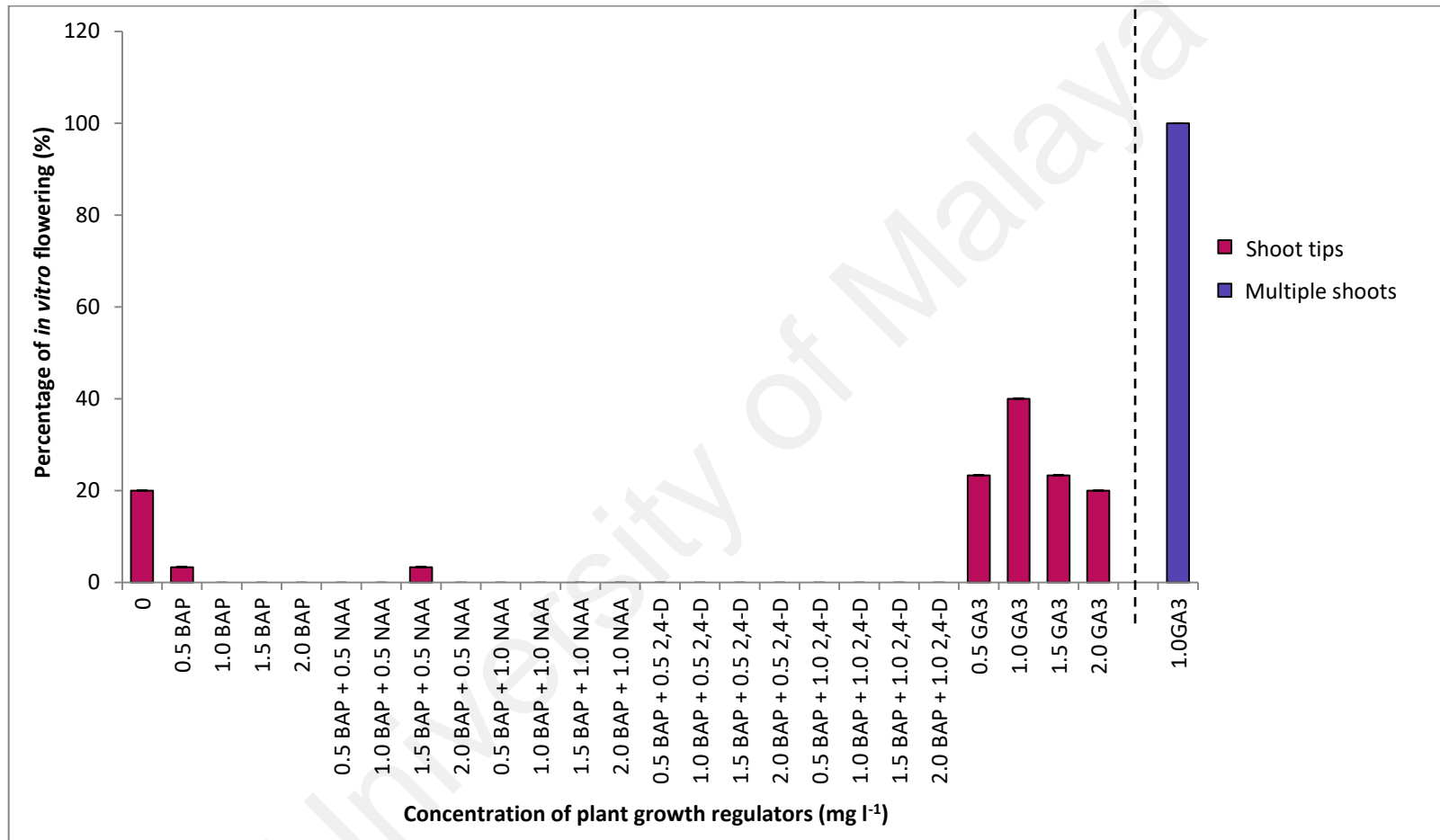
In Setup II, *in vitro* flowering was induced from shoots of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol multiplied on MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP. When transferred to MS medium supplemented with 1.0 mg l<sup>-1</sup> GA<sub>3</sub>, flower buds initiated and later full bloom flowers formed from the multiple shoots (Figure 4.6). On this medium, 100% of the multiple shoots produced normal flowers *in vitro* with a mean of 14 flowers per shoot, before the 16<sup>th</sup> week of culture (Table 4.2). The multiple shoots produced significantly higher percentage of normal *in vitro* flowering as compared to the shoot tip explants. No abnormalities were detected in the flowers developed from the multiple shoots.

**Table 4.2:** *In vitro* flowering from multiple shoots of *O. basilicum* cultured on MS medium supplemented with different PGRs, maintained at 25 ± 2°C under 16 hours of light and 8 hours of darkness, with an irradiance of 50 µmol m<sup>-2</sup> s<sup>-1</sup>.

| Setup II: <i>In vitro</i> flowering induced from multiple shoots |       |   |   |
|--|-------|---|---|
| Concentration of PGRs (mg l <sup>-1</sup> )                      |       | Mean normal <i>in vitro</i> flowering (%) ± SE <sup>y</sup> | Mean abnormal <i>in vitro</i> flowering (%) ± SE <sup>z</sup> |
| Gibberellin  | Auxin |   |   |
| 1.0 GA <sub>3</sub>  | 0     | 100.00 ± 0.00 <sub>a</sub>                                  | 0.00 ± 0.00 <sub>a</sub>                                      |

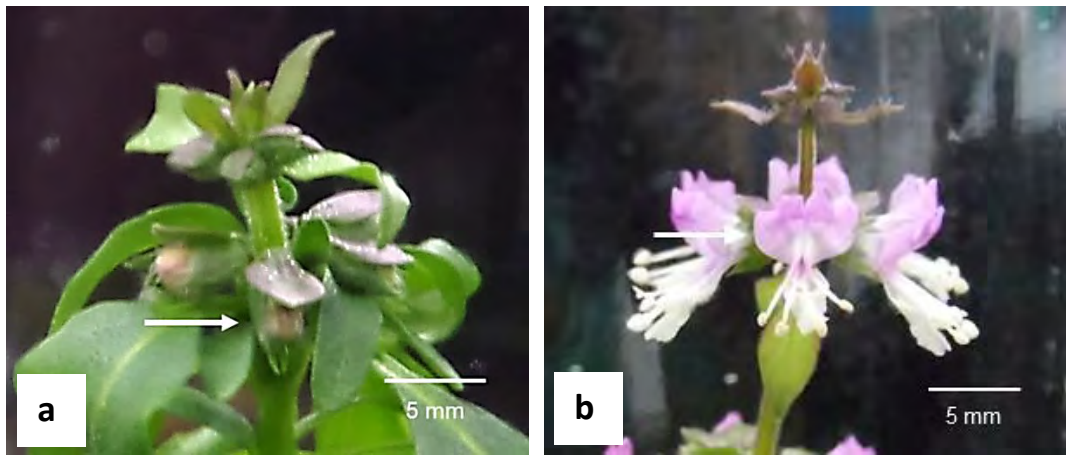
*In vitro* flowering was evaluated until the 16<sup>th</sup> week of culture with 30 shoots per treatment.

<sup>y</sup>, <sup>z</sup> – Values represent mean (%) ± SE of the mean followed by the same letter within columns are not significantly different at *P* = 0.05 by DMRT.



**Figure 4.1:** Effect of concentration of PGRs on the percentage of *in vitro* flowering from shoot tip explants and multiple shoots of *O. basilicum* cultured on MS medium. Error bars are calculated as SE of the mean.

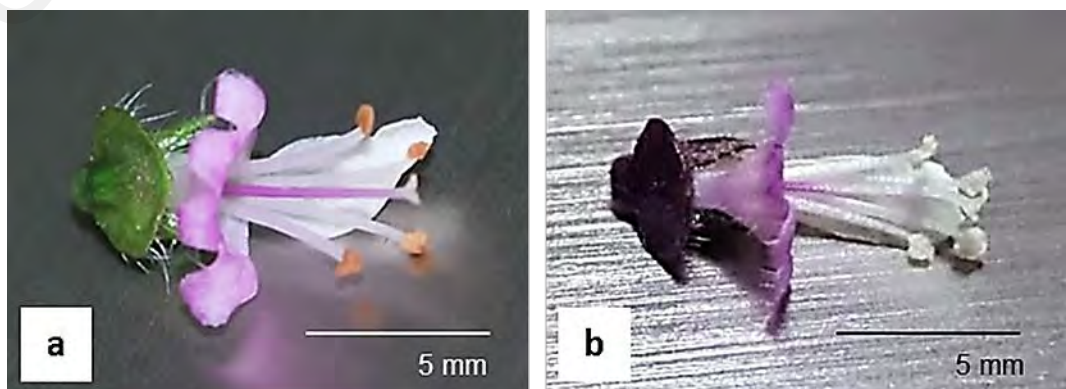




**Figure 4.2:** Shoot tip of *O. basilicum* showing flower bud initiation (*arrow*) after 5 weeks of culture (*a*) and flower formation (*arrow*) after 6 weeks of culture (*b*) on MS medium supplemented with  $1.0 \text{ mg l}^{-1} \text{ GA}_3$ .



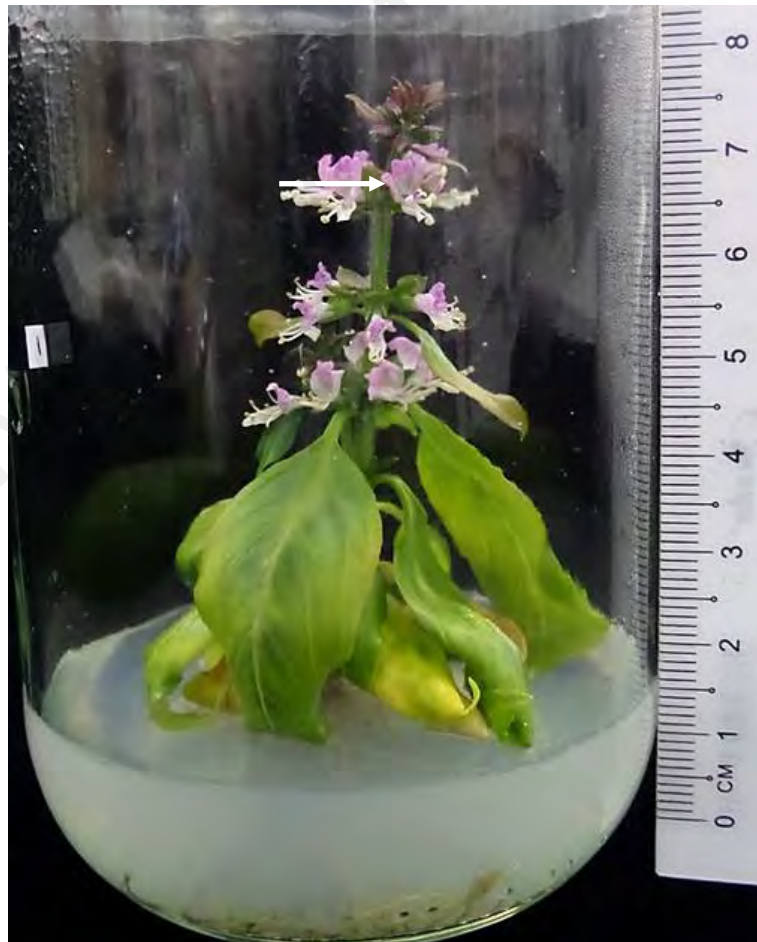
**Figure 4.3:** Formation of *in vitro* flowers (*arrow*) from shoot tip of *O. basilicum* after 7 weeks of culture on MS medium supplemented with  $1.0 \text{ mg l}^{-1} \text{ GA}_3$ .



**Figure 4.4:** Comparison between *in vitro* flower (*a*) and *in vivo* flower (*b*) of *O. basilicum*.



**Figure 4.5:** Formation of abnormal, premature *in vitro* flowers (arrow) from shoot tip of *O. basilicum* after 4 weeks of culture on MS medium supplemented with  $0.5 \text{ mg l}^{-1} \text{ GA}_3$ .



**Figure 4.6:** Formation of *in vitro* flowers (arrow) from multiple shoots of *O. basilicum* after 8 weeks of culture on MS medium supplemented with  $1.0 \text{ mg l}^{-1} \text{ GA}_3$ .

#### 4.4 Summary of Results

1. Efficient protocols for the induction of *in vitro* flowering from shoot tip explants and multiple shoots of *O. basilicum* have been developed for production of normal flowers.
2. MS medium supplemented with and without PGRs at all concentrations tested did not induce *in vitro* flowering from leaf explants.
3. MS medium supplemented with 1.0 mg l<sup>-1</sup> GA<sub>3</sub> induced the highest percentage of *in vitro* flowering from shoot tip explants, with 40% of explants producing *in vitro* flowers before the 20<sup>th</sup> week of culture.
4. When multiple shoots were transferred to MS medium supplemented with 1.0 mg l<sup>-1</sup> GA<sub>3</sub>, 100% of the multiple shoots produced normal *in vitro* flowers, with a mean of 14 flowers per shoot before the 16<sup>th</sup> week of culture.

## CHAPTER 5: ACCLIMATIZATION AND *EX VITRO* FLOWERING OF

### *Ocimum basilicum* L.

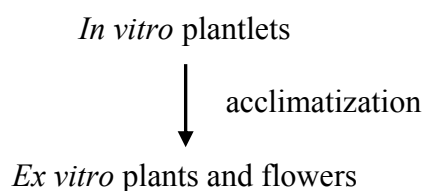
#### 5.1 Objectives of the Experiment

The ultimate success of micropropagation depends on the ability to transfer plantlets out of culture vessels on a large scale with high survival rates. Therefore, it is necessary that the *in vitro* plantlets with well-developed roots are directed to an acclimatization process to ensure that sufficient number of plants survive and grow vigorously when transferred to the natural growing environment in greenhouse or field. This involves gradually moving the plantlets to various substrates that are suitable for their different stages of development. The formation of flowers *ex vitro* might be an indicative of the plant's ability for reproduction.

In this chapter, the objective of the experiment was to measure the efficacy of vermiculite, followed by a soil mixture of three parts garden soil to two parts potting mix to promote acclimatization of *in vitro* regenerated plants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol for establishment and flowering of the plants under *ex vitro* conditions. In this experiment, *in vitro* plantlets of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol were acclimatized in vermiculite while being enclosed in transparent polyethylene bags in a growth chamber before transplanted to a soil mixture (3 garden soil: 2 potting mix) in a field. The percentage of surviving plants was determined.

## 5.2 Materials and Methods

### 5.2.1 Experimental outline



### 5.2.2 Source of *in vitro* plantlets

*In vitro* plantlets of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol produced from direct organogenesis in Chapter 3 were acclimatized for further growth and development under natural environment.

### 5.2.3 Acclimatization and *ex vitro* flowering

After 6 weeks of culture on rooting medium (Section 3.2.5), healthy plantlets with well-developed roots were removed from the rooting medium and the roots were washed gently under running tap water to remove agar. The plantlets were transferred to plastic pots containing moistened vermiculite and covered with transparent polyethylene bags to maintain high humidity (80% to 90% relative humidity). Holes were made in the bags over time to allow gaseous exchange with environment. The plantlets were incubated in a growth chamber maintained at  $25 \pm 2^\circ\text{C}$  under 16 hours of light and 8 hours of darkness, with an irradiance of  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  provided by Philips Lifemax TL-D 18W cool daylight fluorescent tubes.

After 8 weeks, the hardened plantlets were transplanted to polyethylene bags containing a mixture of garden soil and potting mix in a 3:2 ratio. The plants were maintained under the shade with a 12 hours photoperiod provided by sunlight. The

plants were watered once a day and a 15-15-15 nitrogen-phosphorus-potassium (NPK) fertilizer was applied once every 3 weeks. The percentage of surviving plants and mean plant heights were evaluated after 18 weeks of acclimatization (10 weeks after transfer to soil) using thirty plantlets. The acclimatized plants are referred to as the ‘*ex vitro* plants’ and any flowers developed from the acclimatized plants are called the ‘*ex vitro* flowers’. The morphology of the *ex vitro* flowers was compared with the normal *in vivo* flowers which developed from the intact mother plants.

#### **5.2.4 Statistical analysis**

Data for acclimatization was calculated *via* descriptive statistics, using IBM SPSS statistics version 22 software. Results were presented as mean  $\pm$  standard error (SE) of the mean.

## 5.3 Results

### 5.3.1 Acclimatization and *ex vitro* flowering

After 6 weeks of rooting, rooted plantlets of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol were transferred to vermiculite in a growth chamber. Rooted plantlets were successfully acclimatized after 8 weeks of growth in vermiculite (Figure 5.1), and subsequently established in a soil mixture of three parts garden soil to two parts potting mix, in a field under *ex vitro* conditions (Figure 5.2). Eighty percent (80%) of plants survived after 18 weeks of acclimatization, obtaining a mean height of  $25.43 \pm 2.59$  cm (Table 5.1). The *ex vitro* plants were similar in morphology to the *in vivo* plants. After about 24 weeks of acclimatization, normal flowers developed from the *ex vitro* grown plants (Figure 5.3). The *ex vitro* flowers were similar in morphology to the normal *in vivo* flowers (Figure 5.4).

**Table 5.1:** Acclimatization of *O. basilicum*.

| Treatments   | Results   |
|--|---|
| a) Acclimatization in vermiculite, then transplantation in soil (3 garden soil: 2 potting mix) | <ul style="list-style-type: none"><li>• Percentage of surviving plants <math>\pm</math> SE <sup>z</sup>:<br/><math>80.00\% \pm 0.07</math>.</li><li>• Mean plant height <math>\pm</math> SE <sup>z</sup>:<br/><math>25.43 \pm 2.59</math> cm.</li></ul> |

Acclimatization was evaluated after 18 weeks of acclimatization with 30 plantlets per treatment.

<sup>z</sup> – Values represent mean  $\pm$  SE of the mean by descriptive statistics.





**Figure 5.1:** Plantlets of *O. basilicum* after 8 weeks of acclimatization in vermiculite.

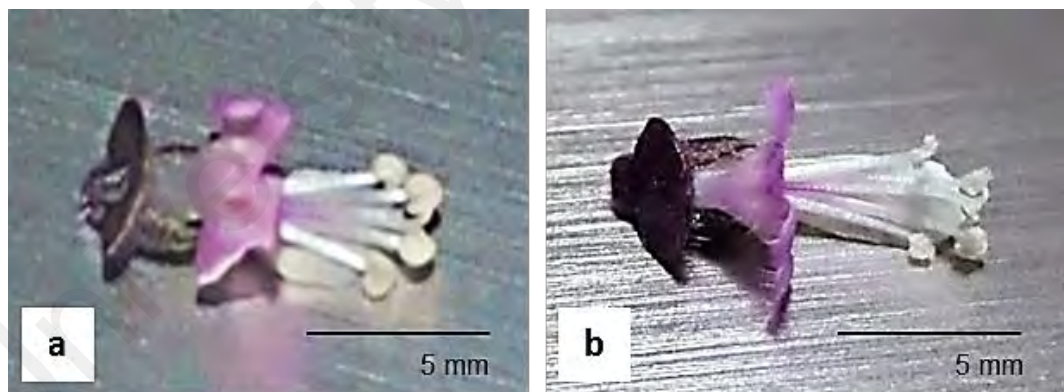


**Figure 5.2:** Acclimatized plants of *O. basilicum* established *ex vitro* 10 weeks after transplant to soil (3 garden soil: 2 potting mix) in the field.





**Figure 5.3:** *Ex vitro* flowers of *O. basilicum* developed after 24 weeks of acclimatization in the field.



**Figure 5.4:** Comparison between *ex vitro* flower (*a*) and *in vivo* flower (*b*) of *O. basilicum*.

#### 5.4 Summary of Results

1. *In vitro* plantlets of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol were successfully acclimatized *ex vitro* for adaptation of the plants under natural growing environment.
2. Eighty percent (80%) of the plantlets survived *ex vitro* after 8 weeks of acclimatization in vermiculite followed by 10 weeks of growth in soil (3 parts garden soil to 2 parts potting mix), obtaining a mean height of  $25.43 \pm 2.59$  cm.
3. The *ex vitro* plants exhibited similar morphology to the *in vivo* plants and showed normal flower development after 24 weeks of acclimatization.

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## CHAPTER 6: POLLEN AND SEED GERMINATION OF *Ocimum basilicum* L.

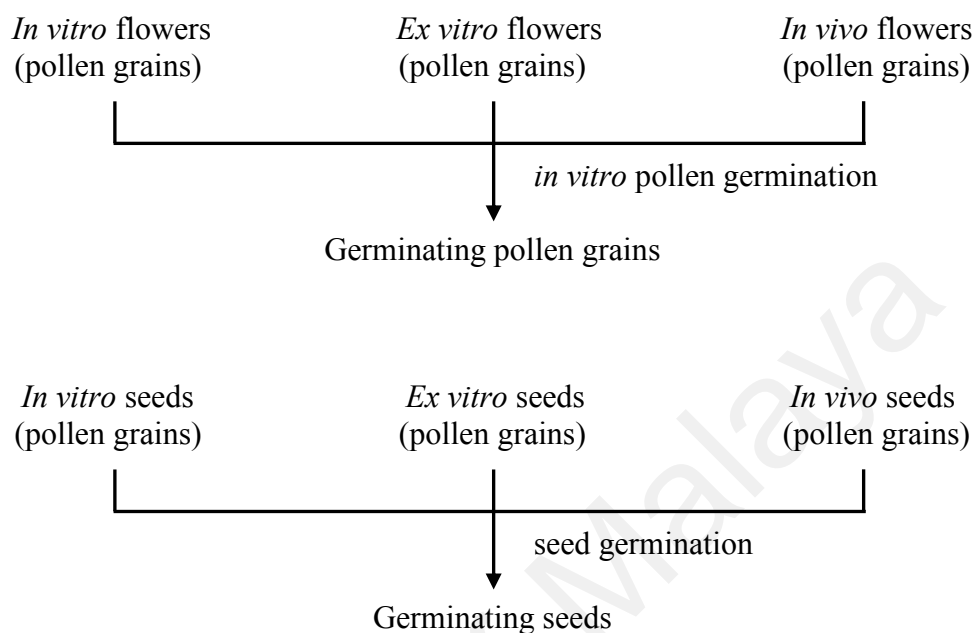
### 6.1 Objectives of the experiment

*In vitro* pollen germination test is a method used to determine the actual germination capability of pollen under suitable conditions, while seed germination test determines the maximum germination potential of the seed. The information is important for helping to enable a greater success rate of crosses and seed formation and therefore enhances the production of a good plant.

In this chapter, the objective of the experiment was to compare the germination rate of pollen grains and seeds between the *in vitro*, *ex vitro*, and *in vivo* grown plants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol for establishment of information on pollen and seed germinability of the plants at different developmental stages. In this experiment, pollen grains of *O. basilicum* were cultured on pollen germination medium containing MS medium, 10% sucrose, and 0.2% gelrite at pH 6.5, and the seeds were germinated on a cotton wool moistened with tap water. The percentages of pollen and seed germination were then calculated.

## 6.2 Materials and Methods

### 6.2.1 Experimental outline



### 6.2.2 Source of pollen grains and seeds

Pollen grains were obtained from the *in vitro*, *ex vitro*, and *in vivo* flowers of *O. basilicum* 'Sweet Thai', methyl chavicol. *In vitro* flowers were the flowers developed on the multiple shoots, maintained under controlled, aseptic growing environment (Chapter 4); *ex vitro* flowers were the flowers developed on the acclimatized plants, after transplanted to the field (Chapter 5); and *in vivo* flowers were the flowers developed on the mother plant, growing under natural environment. The seeds obtained from the *in vitro*, *ex vitro*, and *in vivo* grown plants were then collected, germinated, and estimated for their germination capabilities.

### **6.2.3 *In vitro* pollen germination**

#### **6.2.3.1 Preparation of pollen germination medium**

Pollen germinability was estimated by *in vitro* pollen germination test. To prepare 1 l of pollen germination medium, 100 g of sucrose and 4.4 g of MS powdered medium were dissolved in 700 ml of distilled water in a 1-l conical flask. Then, the volume of the medium was brought up to 1 l with distilled water in a 1-l graduated cylinder, and the pH of the medium was adjusted to  $6.5 \pm 0.1$  using 1.0 N HCl or 1.0 N NaOH. After that, the medium was solidified with 2 g of gelrite and heated until the solution became clear. The medium was autoclaved at 121°C and 15 psi for 20 minutes. After the autoclaving process completed, approximately 10 ml of the medium was dispensed into Petri dishes (85 mm diameter) and let to cool until the gelled medium became semi-solid.

#### **6.2.3.2 *In vitro* germination of pollen grains**

Anthers were collected from *in vitro*, *ex vitro* and *in vivo* flowers during anthesis and the pollen grains were distributed on the germination medium by streaking them over the surface of the medium in petri dishes. All pollen cultures were incubated in a growth chamber maintained at  $25 \pm 2^\circ\text{C}$  in the dark for 48 hours. After that, the pollen grains were observed under light microscope. It was considered germinated when the pollen tubes reached the same or greater length than the pollens' own diameter. The percentage of pollen grains that germinated was determined after 48 hours from a minimum of 100 pollen grains, chosen randomly from five flowers each for *in vitro*, *ex vitro* and *in vivo* grown plants. The *in vivo* pollen grains served as control.

#### **6.2.4 Seed germination**

Seed germinability was estimated by seed germination test. Mature seeds were collected from the *in vitro*, *ex vitro* and *in vivo* grown plants and placed on a layer of cotton wool moistened with tap water in a Petri dish (8.5 cm diameter). All cultures were incubated in a growth chamber maintained at  $25 \pm 2^\circ\text{C}$  under 16 hours of light and 8 hours of darkness, with an irradiance of  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  provided by Philips Lifemax TL-D 18W cool daylight fluorescent tubes. The percentage of seeds that germinated was determined after 21 days from a minimum of 100 seeds, chosen randomly from five plants each for *in vitro*, *ex vitro* and *in vivo* grown plants. The *in vivo* seeds served as control.

#### **6.2.5 Statistical analysis**

Data was statistically analysed using independent-samples Student's t-test for two-group samples or one-way analysis of variance (ANOVA) with confidence intervals calculated via Duncan's multiple range test (DMRT) for more than two-group samples at  $P = 0.05$ , using of IBM SPSS statistics version 22 software. Results were presented as mean  $\pm$  standard error (SE) of the mean.

## 6.3 Results

### 6.3.1 *In vitro* pollen germination

Pollen grains of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol from the *in vitro*, *ex vitro* and *in vivo* flowers showed 0.00%, 43.24% and 41.67% of germination, respectively, by *in vitro* pollen germination test (Table 6.1), but with abnormalities in the pollen tube morphology such as bursting tube (Figure 6.1). The results indicated that the medium failed to induce pollen germination in the *in vitro* flowers; however, it succeeded to induce pollen germination in the *ex vitro* and *in vivo* flowers, which can be detected by the presence of pollen tubes, although with some abnormalities. There was no significant difference in the percentage of pollen germination between the *ex vitro* flowers and the *in vivo* flowers.

### 6.3.2 Seed germination

No seed was formed from the *in vitro* grown plants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol; however some seeds were obtained from the *ex vitro* and *in vivo* grown plants. Each *ex vitro* and *in vivo* flower produced four seeds per calyx. Mature *ex vitro* seeds were black, ovoid in shape, 1.5 to 2 mm in length, and mucilaginous when wet resembling the normal *in vivo* seeds. The seeds formed *ex vitro* and *in vivo* showed 62.26% and 62.50% of germination, respectively by seed germination test (Table 6.1; Figure 6.2). The germination percentage between the two seed sources were not significantly different; hence, the *ex vitro* seeds were as fertile as the *in vivo* seeds.

**Table 6.1:** Comparison of pollen and seed germination percentage between the *in vitro*, *ex vitro*, and *in vivo* grown plants of *O. basilicum*.

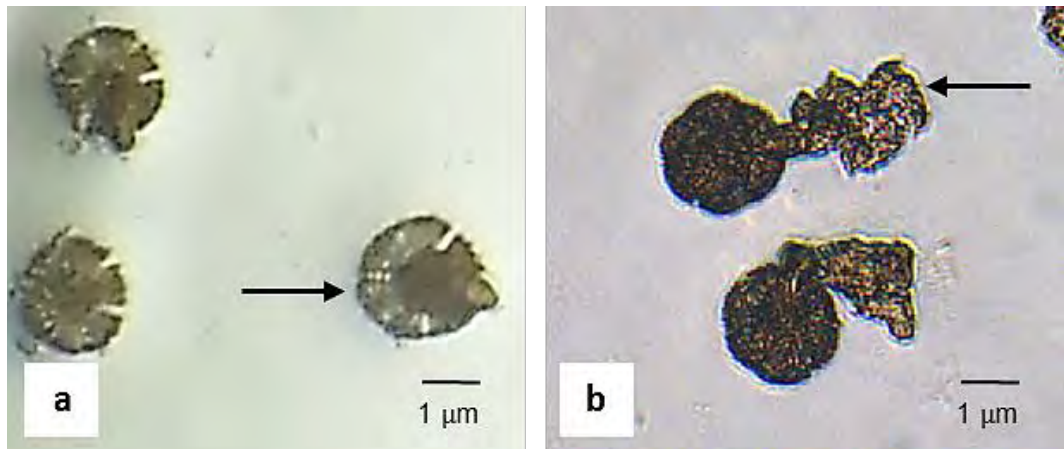
| Experiments   | <i>In vitro</i> plants       | <i>Ex vitro</i> plants        | <i>In vivo</i> plants         |
|---|------------------------------|-------------------------------|-------------------------------|
| a) <i>In vitro</i> pollen germination test.<br>- Pollen germination (%) $\pm$ SE <sup>x</sup> | 0.00 $\pm$ 0.00 <sub>b</sub> | 43.24 $\pm$ 0.05 <sub>a</sub> | 41.67 $\pm$ 0.05 <sub>a</sub> |
| b) Seed germination test.<br>- Seed germination (%) $\pm$ SE <sup>y</sup>                     | No seed formed               | 62.26 $\pm$ 0.05 <sub>a</sub> | 62.50 $\pm$ 0.05 <sub>a</sub> |

<sup>x</sup> – Data was evaluated on pollen grains during anthesis with a minimum of 100 pollen grains per group. Values represent percent mean  $\pm$  SE of the mean followed by the same letter within rows are not significantly different at  $P = 0.05$  by DMRT.

<sup>y</sup> – Data was evaluated on matured seeds with a minimum of 100 seeds per group. Values represent percent mean  $\pm$  SE of the mean followed by the same letter within rows are not significantly different at  $P = 0.05$  by Student's t-test.

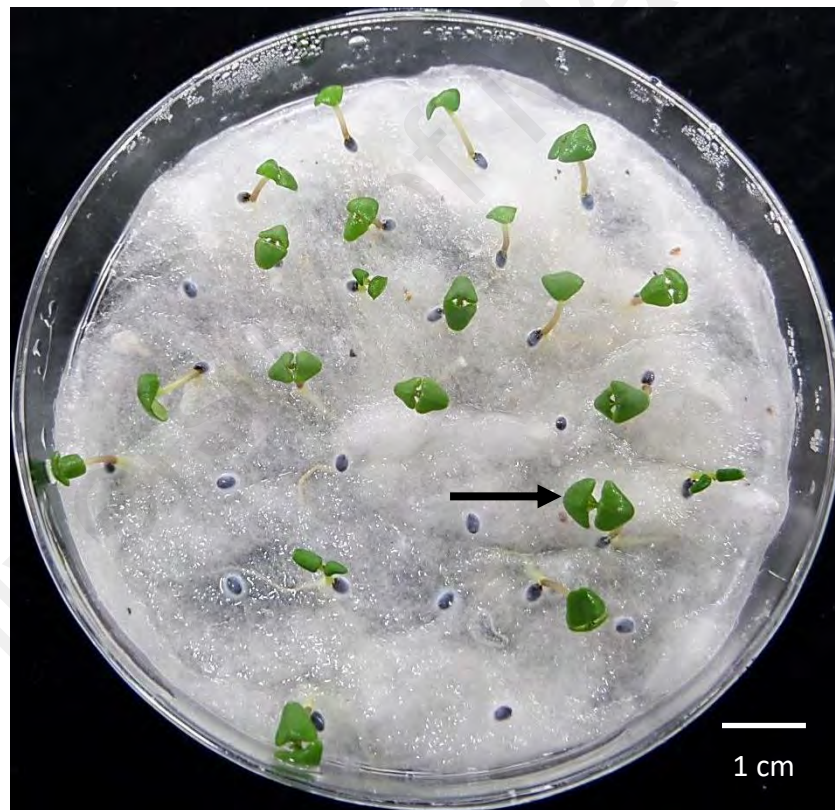
University of Malaysia





**Figure 6.1:**

- (a) Non-germinating pollen grains (*arrow*) from *in vitro* flowers of *O. basilicum*.
- (b) Germinating pollen grains with bursting pollen tube (*arrow*) from *ex vitro* flowers of *O. basilicum*.



**Figure 6.2:** Germinating seeds with first leaf pair (*arrow*) from *ex vitro* grown plants of *O. basilicum*.

#### 6.4 Summary of Results

1. The germination rate of pollen grains and seeds from the *in vitro*, *ex vitro*, and *in vivo* grown plants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol was determined for establishment of information on pollen and seed germinability of the plants at different developmental stages.
2. Pollen grains of the *in vitro* flowers did not germinate in the pollen germination medium, and no seed was formed from the *in vitro* plants.
3. Pollen grains of the *ex vitro* flowers showed a 43.24% germination rate by *in vitro* pollen germination test, which did not differ significantly to the *in vivo* flowers (41.67% germination).
4. Seeds derived from the *ex vitro* grown plants showed a 62.26% germination rate by seed germination test, which did not differ significantly to the *in vivo* grown mother plant (62.50% germination).
5. *Ex vitro* grown plants had the same pollen and seed germinability with the *in vivo* mother plant.

## CHAPTER 7: NON-GLANDULAR AND GLANDULAR TRICHOME

### ULTRASTRUCTURE OF *Ocimum basilicum* L.

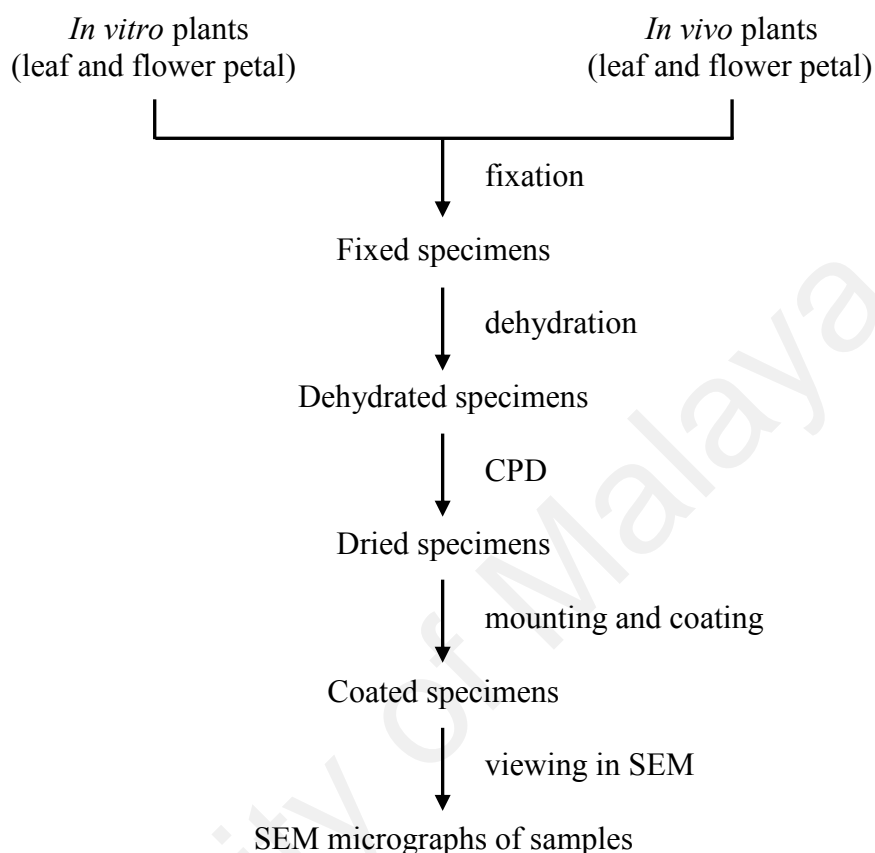
#### 7.1 Objectives of the Experiment

In aromatic plants, the surfaces of leaves and other plant organs are commonly covered by numerous non-glandular and glandular trichomes. Ultrastructural study defines detailed structure of the non-glandular and glandular trichomes, their distribution, density, sizes, and shapes on the plant organ surfaces, and explains their functional importance to the plant.

In this chapter, the objective of the experiment was to compare the distribution, shape, and density of non-glandular and glandular trichomes on leaves and flowers between the *in vitro* and *in vivo* grown plants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol for better understanding on the relationship between their morphology and the plant developmental stages. In this experiment, leaves and flower petals of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol were prepared for scanning electron microscopy (SEM) through a series of steps. These include fixation, dehydration, critical point drying (CPD), mounting, and coating. The prepared samples were then viewed in SEM for ultrastructural examination.

## 7.2 Materials and Methods

### 7.2.1 Experimental outline



### 7.2.2 Source of leaf and flower specimens

Leaves of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol were obtained from three-month-old *in vitro* and *in vivo* plants, while flower petals were obtained from mature *in vitro* and *in vivo* plants during anthesis (full bloom). *In vitro* grown plants used were the multiple shoots, which were maintained under controlled, aseptic growing environment (Chapter 3), and the flowers developed on the *in vitro* grown plants were referred to as the *in vitro* flowers (Chapter 4). *In vivo* grown plants were the mother plants, living under natural growing environment, and the flowers developed on the *in vivo* grown plants were referred to as the *in vivo* flowers.

### 7.2.3 Sample preparation for SEM examination

The third pair of leaves (5 x 5 mm<sup>2</sup> area) from three-month-old plants and the upper lip of corollas (flower petals) during anthesis were fixed in 4% glutaraldehyde in Sörensen's phosphate buffer, pH 7 for one hour at room temperature, then washed in Sörensen's phosphate buffer in distilled water (1:1 v/v buffer/water). Subsequently, the specimens were post-fixed in 1% osmium tetroxide (OsO<sub>4</sub>) in distilled water overnight at low temperature (5°C). Next, the specimens were dehydrated through an ethanol series (from 10% to 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100%, and 100% ethanol), then an ethanol-acetone series (from 3:1 to 1:1, 1:3, and 0:1 v/v ethanol/acetone). Each step had a duration of 15 minutes. Then, the specimens were critical-point dried. By placing the specimens in acetone in the chamber of CPD apparatus, liquid carbon dioxide (CO<sub>2</sub>) replaced the acetone and slowly being heated and drained from the chamber and specimens as a gas. Finally, the critical-point dried specimens were mounted on stubs and dried overnight in a vacuum desiccator before being coated with gold. The prepared samples were viewed in an SEM using JEOL JSM-7001F SEM (JEOL Ltd., Tokyo, Japan), focusing on the non-glandular and glandular trichomes on the adaxial (upper) and abaxial (lower) surfaces of the leaves, and the adaxial (inner) and abaxial (outer) surfaces of the upper lip of corollas from the *in vitro* and *in vivo* grown plants. Five replicates (observation areas) of 1 mm<sup>2</sup> per sample were chosen randomly to evaluate the glandular trichome ultrastructure for each plant type.

### 7.2.4 Statistical analysis

Data was statistically analysed using independent-samples Student's t-test with confidence intervals calculated at  $P = 0.05$ , using IBM SPSS statistics version 22 software. Results were presented as mean  $\pm$  standard error (SE) of the mean.

## 7.3 Results

### 7.3.1 Non-glandular and glandular trichome ultrastructure of the leaves

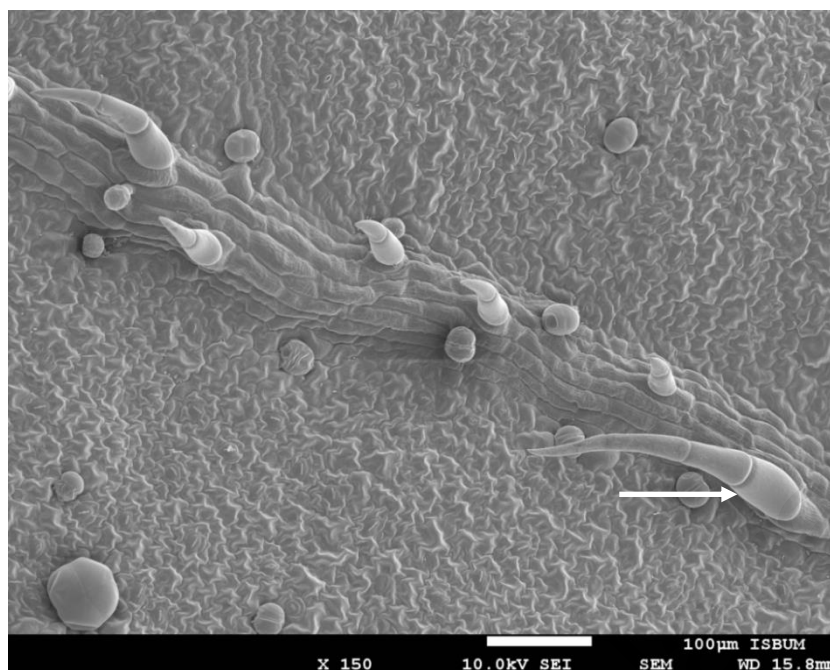
Ultrastructural observations through SEM showed that two types of trichomes were found on the leaves of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol, which were non-glandular trichomes and glandular trichomes. The non-glandular trichomes were pointed, uniseriate, and multicellular of two to five cells (Figure 7.1). The glandular trichomes were divided into capitate glands and peltate glands. Capitate glands composed of a base, a stalk, and a two-celled head, while peltate glands composed of a base, a stalk, and a four-celled head (Figure 7.2).

For leaves, both the *in vitro* and *in vivo* grown plants bore non-glandular trichomes on the abaxial surface, and glandular trichomes on the adaxial and abaxial surfaces. On the abaxial surface, the non-glandular trichomes were arranged on the leaf veins. On the adaxial and abaxial surfaces, the glandular trichomes were scattered over the leaf surfaces. As demonstrated in Table 7.1, using the third pair of leaves from three-month-old plants, it was found that on both sides of leaves, there was no significant difference in the number of peltate and capitate glands per leaf area between the leaves of *in vitro* and *in vivo* grown plants, except for fewer capitate glands on the adaxial surface of *in vivo* leaves. However, the leaves from the *in vitro* grown plants contained fewer peltate glands with fully-filled oil sacs compared to the *in vivo* grown plants (Figure 7.3). The fully-filled oil sacs appeared distended and smooth, while the partially-filled oil sacs appeared creased and wrinkled.

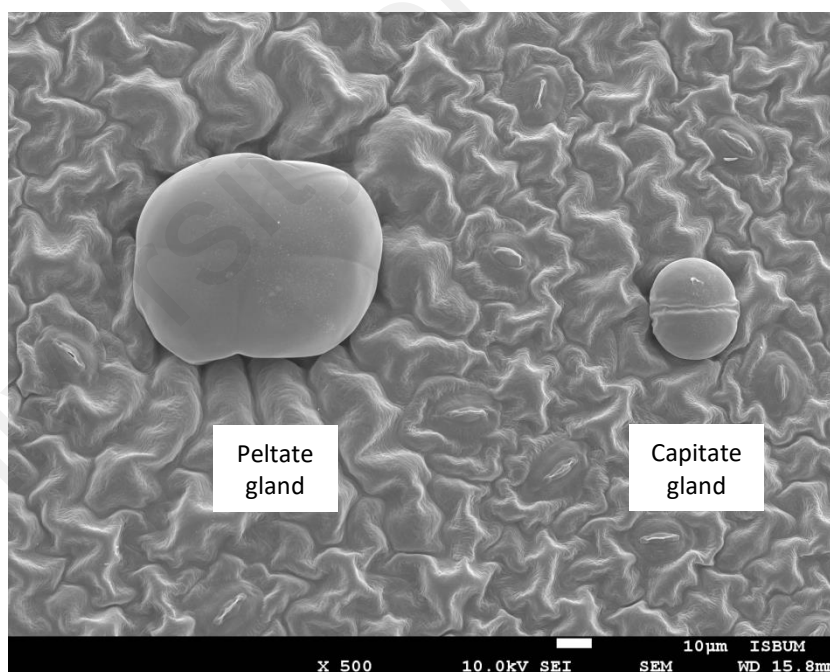
**Table 7.1:** Comparison of non-glandular and glandular trichome ultrastructure on leaves between the *in vitro* and *in vivo* grown plants of *O. basilicum*.

| Results  | <i>In vitro</i> plants <sup>z</sup>  | <i>In vivo</i> plants <sup>z</sup>   |
|--|--|--|
| <p>a) Non-glandular trichomes</p> <ul style="list-style-type: none"> <li>• Structure</li> <li>• Distribution</li> </ul>  | <ul style="list-style-type: none"> <li>• Pointed, uniseriate, and multicellular of two to five cells.</li> <li>• Arranged on the leaf veins on the abaxial surface.</li> </ul>   | <ul style="list-style-type: none"> <li>• Pointed, uniseriate, and multicellular of two to five cells.</li> <li>• Arranged on the leaf veins on the abaxial surface.</li> </ul>   |
| <p>b) Glandular trichomes</p> <ul style="list-style-type: none"> <li>• Structure</li> <li>• Shape</li> <li>• Distribution</li> <li>• Mean number of peltate glands on 1 mm<sup>2</sup> of leaf (adaxial surface) ± SE<sup>y</sup></li> <li>• Mean number of peltate glands on 1 mm<sup>2</sup> of leaf (abaxial surface) ± SE<sup>y</sup></li> <li>• Mean number of capitate glands on 1 mm<sup>2</sup> of leaf (adaxial surface) ± SE<sup>y</sup></li> <li>• Mean number of capitate glands on 1 mm<sup>2</sup> of leaf (abaxial surface) ± SE<sup>y</sup></li> </ul> | <ul style="list-style-type: none"> <li>• Capitate glands: a base, a stalk, and a two-celled head.</li> <li>• Peltate glands: a base, a stalk, and a four-celled head.</li> <li>• Mostly creased and wrinkled.</li> <li>• Scattered over the adaxial and abaxial surfaces.</li> <li>• 8.00 ± 0.77<sub>a</sub></li> <li>• 6.80 ± 0.49<sub>a</sub></li> <li>• 19.60 ± 0.75<sub>a</sub></li> <li>• 20.00 ± 0.71<sub>a</sub></li> </ul> | <ul style="list-style-type: none"> <li>• Capitate glands: a base, a stalk, and a two-celled head.</li> <li>• Peltate glands: a base, a stalk, and a four-celled head.</li> <li>• Mostly distended and smooth.</li> <li>• Scattered over the adaxial and abaxial surfaces.</li> <li>• 8.40 ± 0.51<sub>a</sub></li> <li>• 6.20 ± 0.37<sub>a</sub></li> <li>• 15.80 ± 1.02<sub>b</sub></li> <li>• 17.60 ± 0.98<sub>a</sub></li> </ul> |

<sup>z</sup> – Data was evaluated on the third pair of leaves from 3-month-old *in vitro* and *in vivo* grown plants with 5 replicates per group. Values represent percent mean ± SE of the mean followed by the same letter within rows are not significantly different at  $P = 0.05$  by Student's t-test.

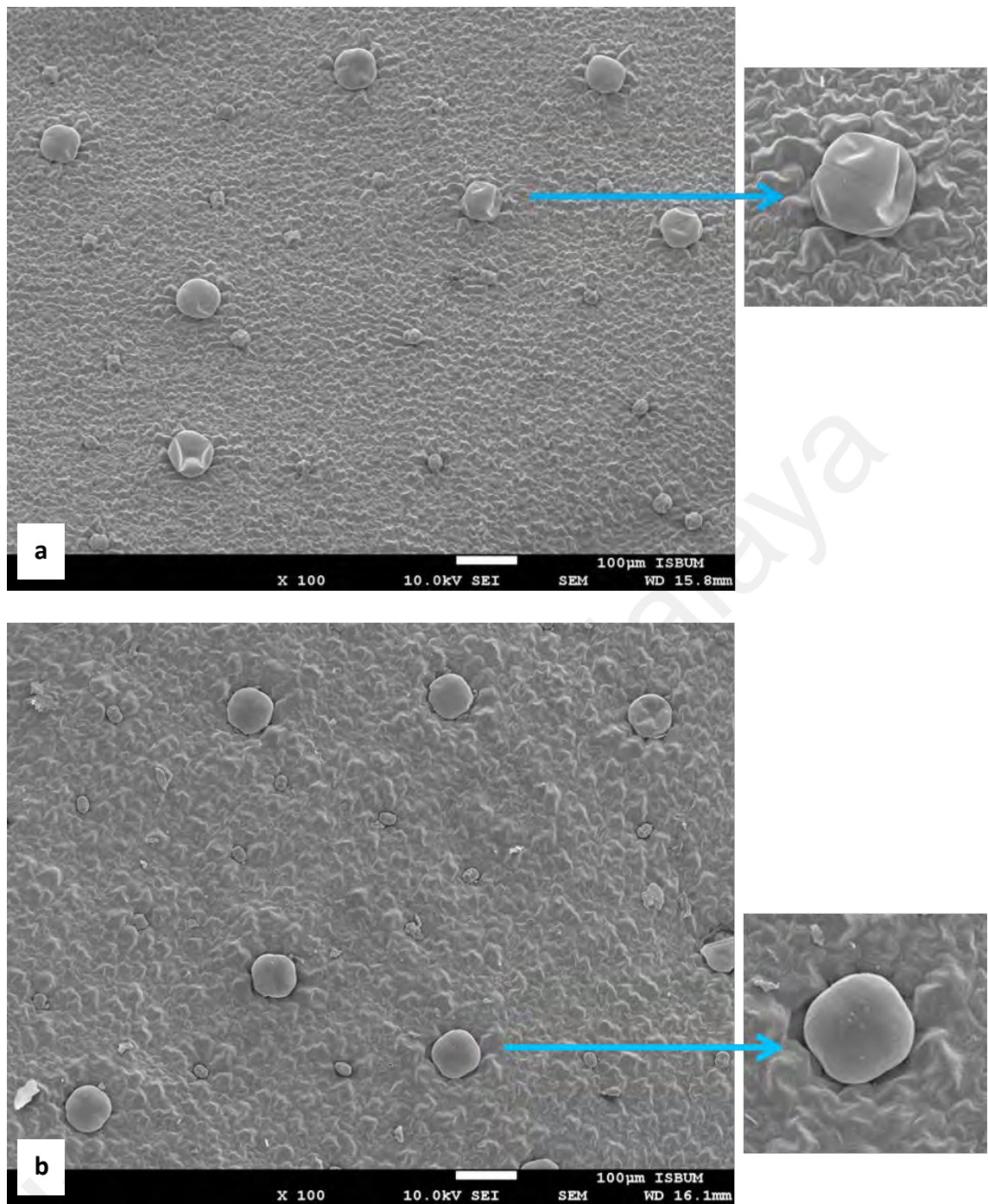


**Figure 7.1:** SEM micrograph from the abaxial surface of an *in vitro* leaf of *O. basilicum*, showing non-glandular trichomes (arrow) were arranged on the leaf veins (bar = 100µm).



**Figure 7.2:** SEM micrograph from the abaxial surface of an *in vitro* leaf of *O. basilicum*, showing glandular trichomes were divided into two-celled head capitate gland and four-celled head peltate gland (bar = 10µm).





**Figure 7.3:** SEM micrographs of an *in vitro* (a) and *in vivo* (b) leaf of *O. basilicum*, showing glandular trichomes were scattered on the abaxial surface.

- (a) Most of the peltate glands on the *in vitro* leaf appeared creased and wrinkled (*bar* = 100μm).
- (b) Most of the peltate glands on the *in vivo* leaf appeared distended and smooth (*bar* = 100μm).

### 7.3.2 Non-glandular and glandular trichome ultrastructure of the flowers

Ultrastructural SEM observations indicated that two types of trichomes were found on the upper lip of corollas of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol, which were non-glandular trichomes and glandular trichomes. The non-glandular trichomes were pointed, uniseriate, and multicellular of two to five cells. The glandular trichomes were divided into capitate glands and peltate glands. Capitate glands composed of a base, a stalk, and a two-celled head, while peltate glands composed of a base, a stalk, and a four-celled head.

For upper lip of corollas, both the *in vitro* and *in vivo* grown plants bore non-glandular trichomes on the adaxial and abaxial surfaces (Table 7.2). On the adaxial surface, the non-glandular trichomes were situated at the base of corollas, forming on the inner surface of the corolla tubes a villous indumentum; while on the abaxial surface, the non-glandular trichomes were mixed together with the glandular trichomes, concentrated at the tip of corollas near the corolla lobes (Figure 7.4). Most of the non-glandular trichomes on the *in vitro* flowers appeared creased and wrinkled, while most of the non-glandular trichomes on the *in vivo* flowers appeared distended and smooth (Figure 7.5).

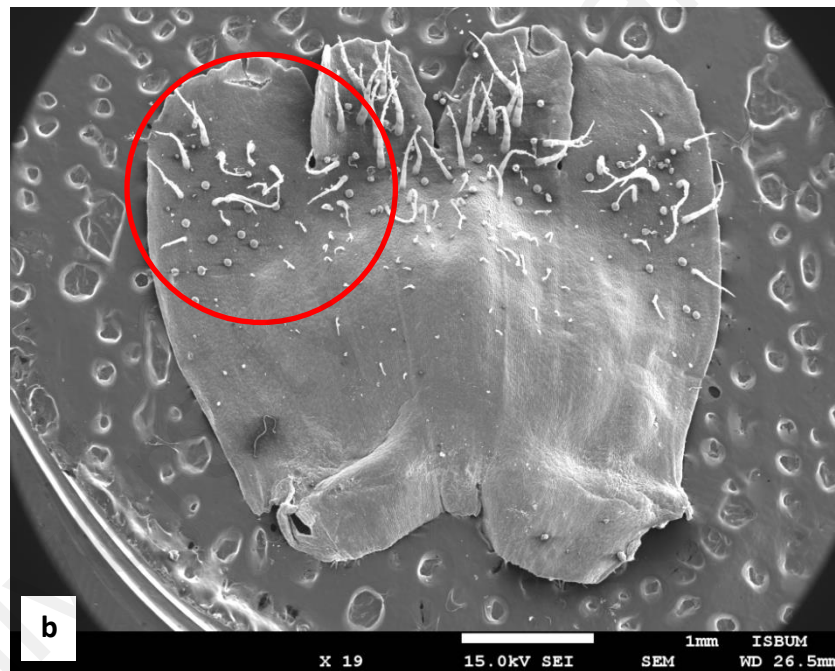
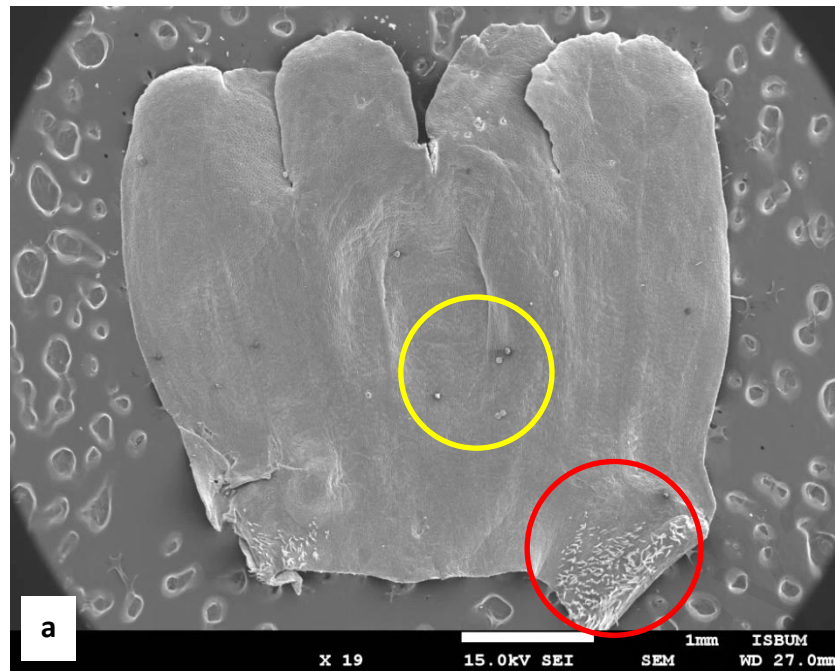
Upper lip of corollas from the *in vitro* grown plants bore glandular trichomes on the abaxial surface only; however upper lip of corollas from the *in vivo* grown plants bore glandular trichomes on the adaxial and abaxial surfaces. On the adaxial surface, the glandular trichomes were scattered in the centre of corollas; while on the abaxial surface, the glandular trichomes were concentrated at the tip of corollas near the corolla lobes. Most of the glandular trichomes on the *in vitro* flowers appeared creased and

wrinkled, while most of the glandular trichomes on the *in vivo* flowers appeared distended and smooth (Figure 7.6).

**Table 7.2:** Comparison of non-glandular and glandular trichome ultrastructure on upper lip of corollas between the *in vitro* and *in vivo* grown plants of *O. basilicum*.

| Results  | <i>In vitro</i> plants <sup>z</sup>  | <i>In vivo</i> plants <sup>z</sup>  |
|--|--|---|
| <p>a) Non-glandular trichomes</p> <ul style="list-style-type: none"> <li>• Structure</li> <li>• Shape</li> <li>• Distribution</li> </ul> | <ul style="list-style-type: none"> <li>• Pointed, uniseriate, and multicellular of two to five cells.</li> <li>• Mostly creased and wrinkled.</li> <li>• At the base of corolla on the adaxial surface, and at the tip of corolla on the abaxial surface.</li> </ul>                           | <ul style="list-style-type: none"> <li>• Pointed, uniseriate, and multicellular of two to five cells.</li> <li>• Mostly distended and smooth.</li> <li>• At the base of corolla on the adaxial surface, and at the tip of corolla on the abaxial surface.</li> </ul>  |
| <p>b) Glandular trichomes</p> <ul style="list-style-type: none"> <li>• Structure</li> <li>• Shape</li> <li>• Distribution</li> </ul>     | <ul style="list-style-type: none"> <li>• Capitate glands: a base, a stalk, and a two-celled head.</li> <li>• Peltate glands: a base, a stalk, and a four-celled head.</li> <li>• Mostly creased and wrinkled.</li> <li>• Concentrated at the tip of corolla on the abaxial surface.</li> </ul> | <ul style="list-style-type: none"> <li>• Capitate glands: a base, a stalk, and a two-celled head.</li> <li>• Peltate glands: a base, a stalk, and a four-celled head.</li> <li>• Mostly distended and smooth.</li> <li>• At the centre of corolla on the adaxial surface, and concentrated at the tip of corolla on the abaxial surface.</li> </ul> |

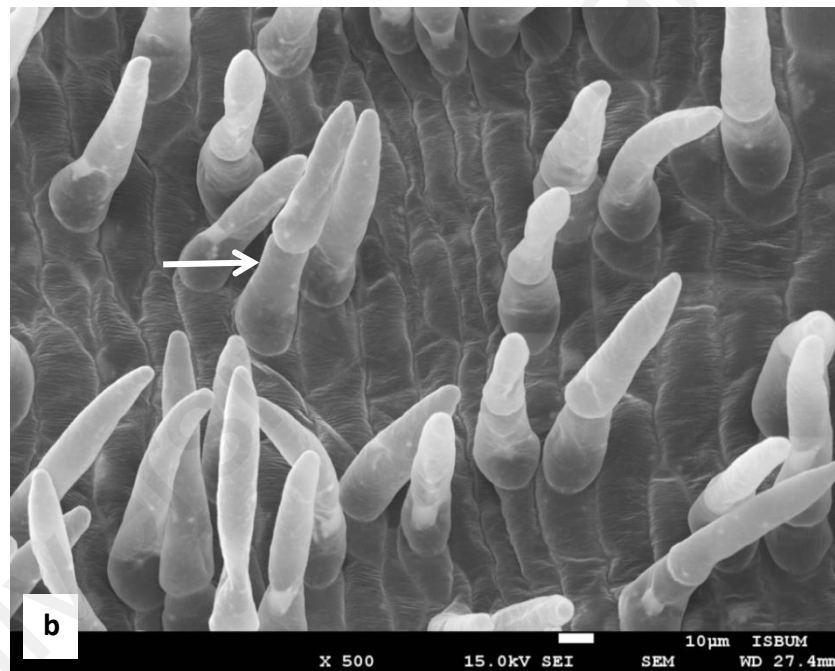
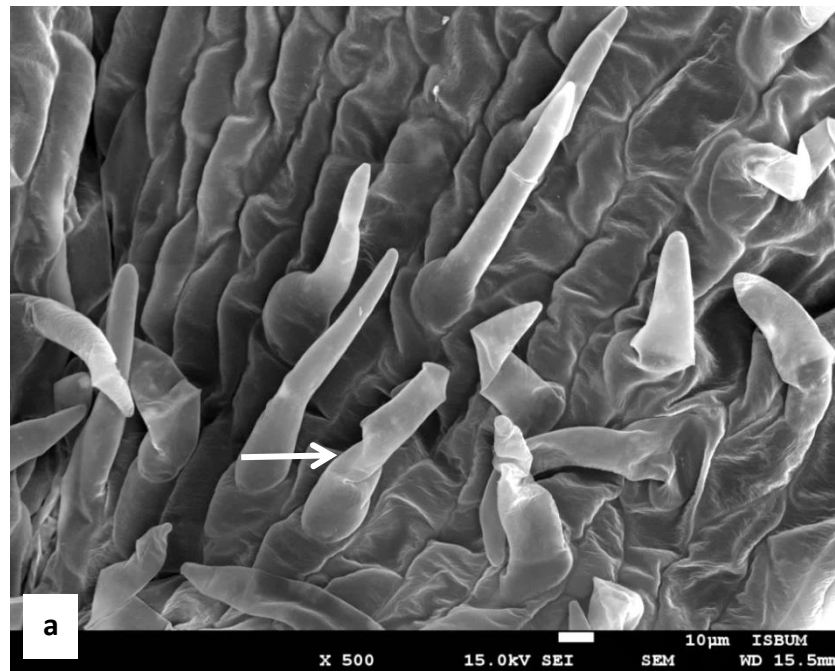
<sup>z</sup> – Data was evaluated on the upper lip of corollas during anthesis from the *in vitro* and *in vivo* grown plants with 5 replicates per group.



**Figure 7.4:** SEM micrographs of an *in vivo* flower of *O. basilicum*, showing non-glandular and glandular trichomes on the adaxial (a) and abaxial (b) surfaces.

(a) On the adaxial surface, non-glandular trichomes (red circle) were at the base and glandular trichomes (yellow circle) were in the centre of the corolla upper lip (bar = 1 mm).

(b) On the abaxial surface, non-glandular and glandular trichomes (red circle) were mixed, concentrated at the tip of the corolla upper lip (bar = 1 mm).

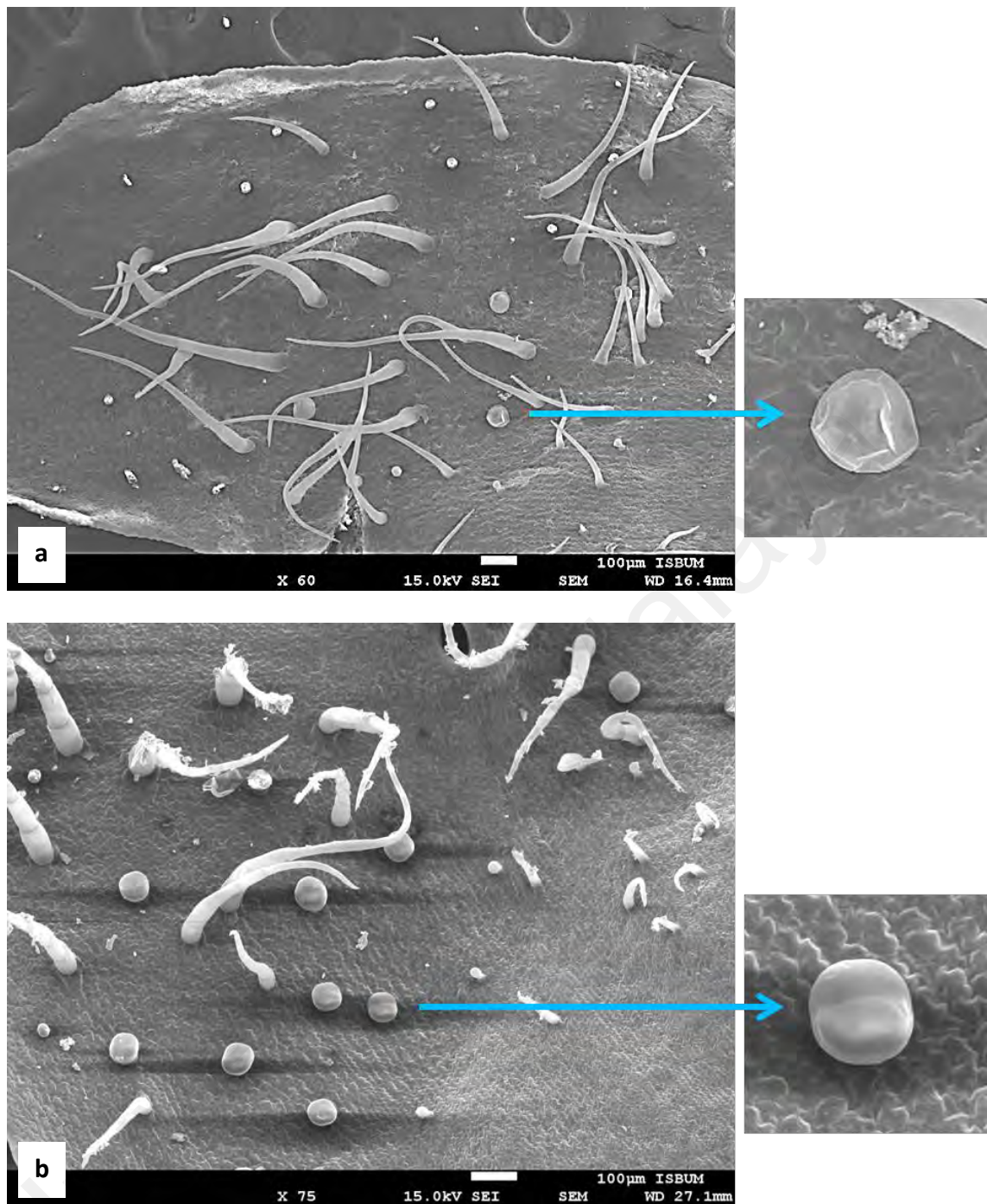


**Figure 7.5:** SEM micrographs of an *in vitro* (a) and *in vivo* (b) flower of *O. basilicum*, showing non-glandular trichomes on the adaxial surface of corolla upper lip.

(a) Most of the non-glandular trichomes (arrow) on the *in vitro* flower appeared creased and wrinkled (bar = 10µm).

(b) Most of the non-glandular trichomes (arrow) on the *in vivo* flower appeared distended and smooth (bar = 10µm).





**Figure 7.6:** SEM micrographs of an *in vitro* (a) and *in vivo* (b) flower of *O. basilicum*, showing glandular trichomes on the abaxial surface of corolla upper lip.

- (a) Most of the glandular trichomes on the *in vitro* flower appeared creased and wrinkled (*bar* = 100µm).
- (b) Most of the glandular trichomes on the *in vivo* flower appeared distended and smooth (*bar* = 100µm).

#### 7.4 Summary of Results

1. The distribution, shape, and density of non-glandular and glandular trichomes on leaves and flowers from the *in vitro* and *in vivo* grown plants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol were determined for better understanding on the relationship between their morphology and the plant developmental stages.
2. Leaves from both the *in vitro* and *in vivo* grown plants bore non-glandular trichomes on the leaf veins of the abaxial surface and glandular trichomes over the adaxial and abaxial surfaces.
3. Upper lip of corollas from both the *in vitro* and *in vivo* grown plants bore non-glandular trichomes at the base of corollas on the adaxial surface and at the tip of corollas on the abaxial surface.
4. Upper lip of corollas from the *in vitro* grown plants bore glandular trichomes at the tip of corollas on the abaxial surface only, but upper lip of corollas from the *in vivo* grown plants bore glandular trichomes in the centre of corollas on the adaxial surface and at the tip of corollas on the abaxial surface.
5. On both sides of leaves, there was no significant difference in the number of peltate and capitate glands per leaf area between the *in vitro* and *in vivo* grown plants, except for fewer capitate glands on the adaxial surface of *in vivo* leaves.
6. Most of the non-glandular and glandular trichomes on the *in vitro* leaves and upper lip of corollas appeared creased and wrinkled; whereas most of the non-glandular and glandular trichomes on the *in vivo* leaves and upper lip of corollas appeared distended and smooth.

## CHAPTER 8: ESSENTIAL OIL ACCUMULATION IN *Ocimum basilicum* L.

### 8.1 Objectives of the Experiment

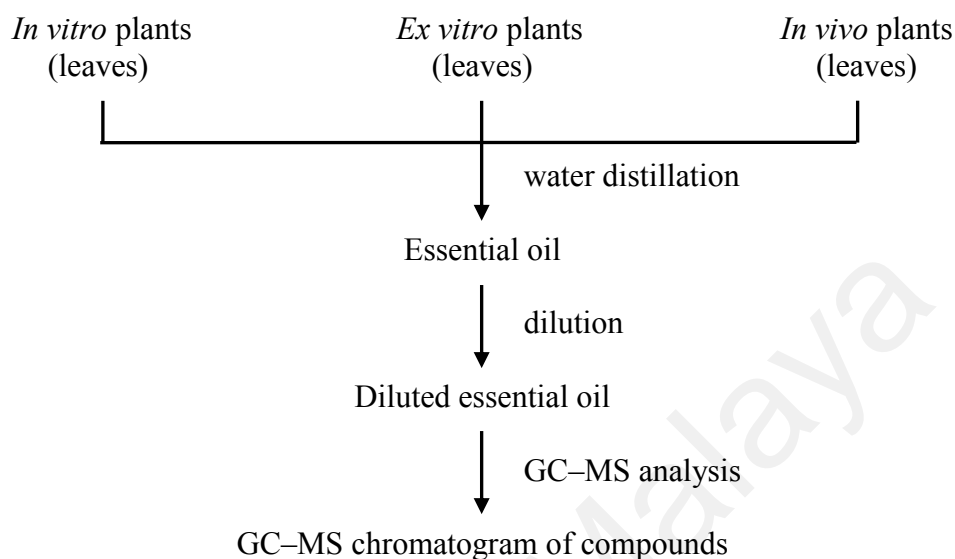
Aromatic plants usually contain essential oils in their organs. Essential oil is a natural oil typically obtained by distillation and having the characteristic odour of the plant from which it is extracted. The essential oil content and composition in plants vary at different stages of plant development.

In this chapter, the objective of the experiment was to compare the essential oil content and composition in leaves between the *in vitro*, *ex vitro* and *in vivo* grown plants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol for establishment of information on essential oil and compound accumulation of the plants at different developmental stages. In this experiment, essential oils were extracted from the *in vitro*, *ex vitro*, and *in vivo* grown plants of *O. basilicum* by water distillation method, and the essential oils obtained were analysed for the identification and quantification of their compounds by gas chromatography–mass spectrometry (GC–MS).



## 8.2 Materials and Methods

### 8.2.1 Experimental outline



### 8.2.2 Source of essential oils

Essential oil of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol, obtained from leaf part of the plant, was analysed by GC-MS. Analyzed plants were at the following physiological stages: *In vitro* grown plants – multiple shoots, after 20 weeks of culture under controlled, aseptic growing environment (Chapter 3); *ex vitro* grown plants – acclimatized plants, after 18 weeks of transfer from culture medium to soil (Chapter 5); and *in vivo* grown plants – mature mother plant, prior to flowering, growing under natural environment.

### 8.2.3 Extraction of essential oils

Essential oils were extracted from leaves by water distillation method. Fresh leaves of about 150 g was collected from the *in vitro*, *ex vitro* and *in vivo* grown plants, and was subjected to a 4-hour water distillation using a Clevenger-type apparatus with heat provided by a Favorit heating mantle (PLT Scientific Sdn. Bhd., Puchong,

Selangor, Malaysia). The oils obtained were weighed, and the yields were expressed relative to the dry mass of leaves. The oil extracts were stored in glass vials at -20°C until analysis.

#### 8.2.4 Analysis of essential oil composition

GC-MS analysis of essential oil was performed using a Shimadzu GC-2010 coupled with a Shimadzu GCMS-QP 2010 Plus (Shimadzu Corp., Kyoto, Japan) equipped with a flame ionization detector (FID). One microliter (1  $\mu$ l) of sample containing 5% essential oil in hexane was injected into a gas chromatograph equipped with DB-5MS capillary column (30 m x 250  $\mu$ m, film thickness 0.25  $\mu$ m) using helium as the carrier gas, with a flow rate of 1 ml min<sup>-1</sup>, injector temperature of 250°C, and a split injection ratio of 10:1. The oven temperature was initially 40°C for 2 min, with temperature increasing at a rate of 3°C min<sup>-1</sup> until it reached 140°C for 2 min, after which the rate of temperature increase was 10°C min<sup>-1</sup> until it reached 250°C, where the temperature was held for 5 min.

#### 8.2.5 Identification of volatile compounds

The Kovats retention indices of compounds in the essential oils were determined based on the alkane series C<sub>6</sub>-C<sub>23</sub>. For temperature programmed chromatography, the Kovats index is given by the equation:

$$I = 100 \times \left[ n + \frac{t_{r(\text{unknown})} - t_{r(n)}}{t_{r(N)} - t_{r(n)}} \right]$$

Where

- $I$  = Kovats retention index;
- $n$  = the number of carbon atoms in the n-alkane eluting before the compound;
- $t_{r(\text{unknown})}$  = the retention time of the compound;
- $t_{r(N)}$  = the retention time of the n-alkane eluting after the compound;
- $t_{r(n)}$  = the retention time of the n-alkane eluting before the compound.

Identification of individual compounds was carried out by comparing their mass spectra with the mass spectra in NIST Mass Spectral Library (NIST, 2008) and by the means of their retention indices, compared with those in the literature (Adams, 2007; Wesolowska *et al.*, 2012; Nurzyńska-Wierdak *et al.*, 2013). Data was given as percentage mass for each compound in the essential oil. *In vitro* grown plants were assessed after 20 weeks of culture, *ex vitro* grown plants were assessed 18 weeks after acclimatization, and *in vivo* grown plants were assessed at maturity, before flowering.

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## 8.3 Results

### 8.3.1 Essential oil content

The highest essential oil content, 4.50%, was found in the *in vivo* grown plants of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol. The essential oil content of the *in vitro* grown plants (1.99%) was initially lower than the *in vivo* grown plants (4.50%), but after 18 weeks of acclimatization, the essential oil content of the *ex vitro* grown plants increased to 2.38% (Table 8.1).

### 8.3.2 Essential oil composition

By GC–MS analysis, each compound in the essential oils of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol was separated and identified, and its relative amount was quantified as percentage. The composition of the essential oils was listed as in Table 8.1. Thirty main compounds were identified in the essential oils of *O. basilicum* leaves.

Methyl chavicol was found as the dominant compound in all the three essential oil samples: *in vitro*, *ex vitro* and *in vivo* grown plants. This dominance can be seen from the largest peak area produced by methyl chavicol in the chromatogram, where the peak area represented the methyl chavicol percentage in the essential oil. Methyl chavicol dominance occurred due to the use of chemotype ‘methyl chavicol’ as the source of *O. basilicum* plant in this study. Although methyl chavicol was dominant in all essential oil samples, its relative quantity differed in varying modes of plant growth. The percentage of methyl chavicol in the *in vitro* grown plants (93.71%) initially was higher than in the *in vivo* grown plants (66.29%), but then the percentage dropped to 60.07% after 18 weeks of the plants being acclimatized (*ex vitro* plants). The amount of

methyl chavicol, both in the *in vivo* and *ex vitro* grown plants, was approximately the same, as the plants were at almost the same developmental stage.

Besides methyl chavicol, the following compounds were found to present in high amounts in the essential oils: methyl eugenol (1.64%), trans- $\beta$ -ocimene (0.75%), and trans- $\beta$ -caryophyllene (0.60%) in the *in vitro* grown plants (Figure 8.1), while trans- $\beta$ -ocimene (8.26–12.83%), 1,8-cineole (3.83–7.04%), and camphor (2.69–3.61%) in both the *ex vitro* and *in vivo* grown plants (Figures 8.2 and 8.3). Their mass spectra were shown in Figure 8.4.

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**Table 8.1:** Essential oil content and composition (%) in *O. basilicum* leaves from *in vitro*, *ex vitro* and *in vivo* grown plants.

|                               |      | <i>In vitro</i> plants<br>(%) <sup>x</sup> | <i>Ex vitro</i> plants<br>(%) <sup>y</sup> | <i>In vivo</i> plants<br>(%) <sup>z</sup> |
|-------------------------------|------|--|--|---|
| Essential oil content (m/m)   |      | 1.99                                       | 2.38                                       | 4.50                                      |
| Compounds                     | KI   |  |  |   |
| $\alpha$ -Pinene              | 942  | 0.05                                       | 0.39                                       | 0.36                                      |
| Sabinene                      | 973  | 0.11                                       | 0.28                                       | 0.23                                      |
| $\beta$ -Pinene               | 977  | 0.03                                       | 0.60                                       | 0.38                                      |
| $\beta$ -Myrcene              | 988  | 0.04                                       | 1.89                                       | 1.36                                      |
| Limonene                      | 1024 | 0.05                                       | 0.45                                       | 0.16                                      |
| 1,8-Cineole                   | 1027 | 0.27                                       | 3.83                                       | 7.04                                      |
| cis- $\beta$ -Ocimene         | 1031 | 0.02                                       | 0.82                                       | 0.54                                      |
| trans- $\beta$ -Ocimene       | 1045 | 0.75                                       | 8.26                                       | 12.83                                     |
| Terpinolene                   | 1080 | 0.07                                       | 0.65                                       | 0.53                                      |
| $\beta$ -Linalool             | 1096 | 0.11                                       | 0.73                                       | 0.80                                      |
| Camphor                       | 1141 | 0.28                                       | 2.69                                       | 3.61                                      |
| Methyl chavicol               | 1196 | 93.71                                      | 60.07                                      | 66.29                                     |
| Bornyl acetate                | 1287 | 0.21                                       | 0.74                                       | 0.17                                      |
| Elixene                       | 1332 | 0.01                                       | 0.54                                       | tr.                                       |
| $\beta$ -Elemene              | 1391 | 0.23                                       | 1.78                                       | 0.02                                      |
| Methyl eugenol                | 1407 | 1.64                                       | 2.50                                       | 0.52                                      |
| trans- $\beta$ -Caryophyllene | 1425 | 0.60                                       | 1.84                                       | 0.49                                      |
| trans- $\alpha$ -Bergamotene  | 1440 | 0.10                                       | 1.50                                       | 0.77                                      |
| $\alpha$ -Guaiene             | 1442 | 0.08                                       | 0.34                                       | 1.07                                      |
| cis- $\beta$ -Farnesene       | 1461 | 0.34                                       | 0.90                                       | 0.51                                      |
| $\alpha$ -Humulene            | 1464 | 0.17                                       | 1.36                                       | 0.22                                      |
| cis-Muurolo-4(14),5-diene     | 1469 | 0.02                                       | 0.51                                       | 0.06                                      |
| Germacrene D                  | 1486 | 0.05                                       | 0.70                                       | 0.08                                      |
| Bicyclogermacrene             | 1497 | 0.11                                       | 0.82                                       | 0.12                                      |
| $\alpha$ -Bulnesene           | 1503 | 0.14                                       | 0.67                                       | 0.17                                      |
| $\gamma$ -Cadinene            | 1516 | 0.15                                       | 1.22                                       | 0.46                                      |
| 1,10-di-epi-Cubenol           | 1611 | 0.06                                       | 0.65                                       | 0.26                                      |
| $\tau$ -Cadinol               | 1646 | 0.52                                       | 2.25                                       | 0.54                                      |
| $\beta$ -Eudesmol             | 1660 | 0.06                                       | 0.61                                       | 0.28                                      |
| $\alpha$ -Bisabolol           | 1692 | 0.02                                       | 0.09                                       | 0.13                                      |
| Others                        |      | 0.00                                       | 0.32                                       | 0.00                                      |
| Total                         |      | 100.00                                     | 100.00                                     | 100.00                                    |

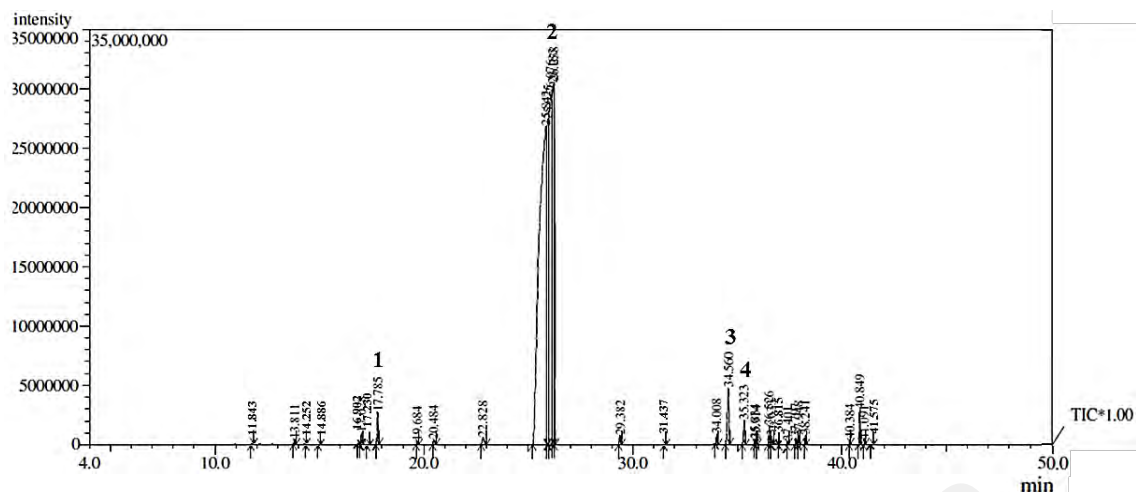
KI Kovats index, relative to n-alkane series calculated on a DB-5MS column.

<sup>x</sup> – Multiple shoots (*in vitro*), after 20 weeks of culture.

<sup>y</sup> – Acclimatized plants (*ex vitro*), after 18 weeks of transfer from culture medium to soil.

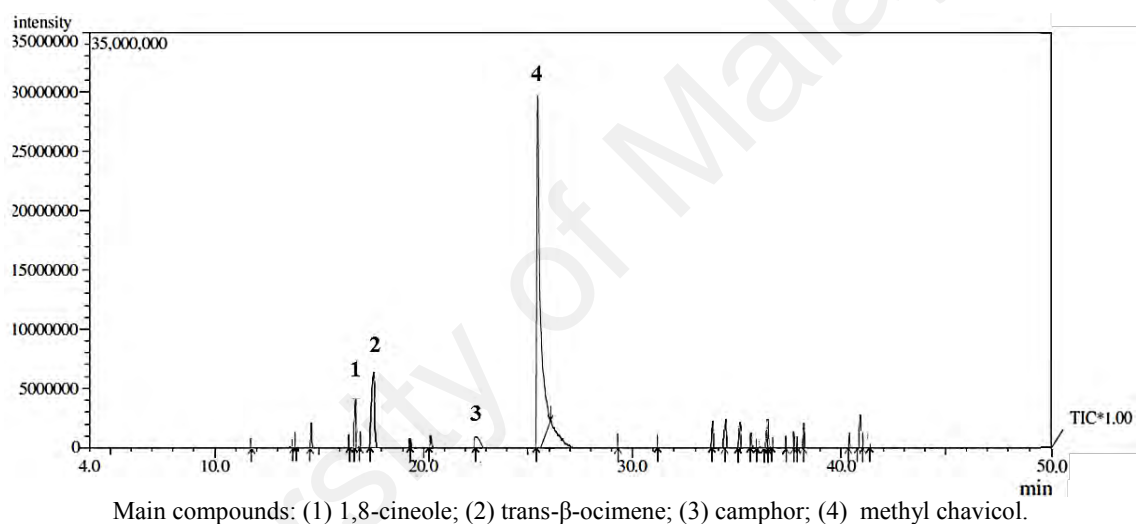
<sup>z</sup> – Mature mother plants (*in vivo*), prior to flowering.

tr. – the compound present in trace amount.



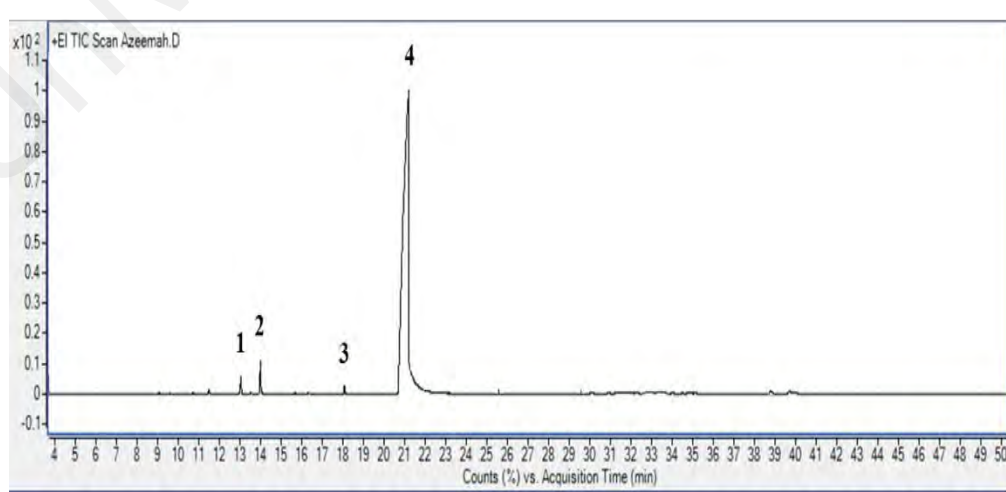
Main compounds: (1) trans- $\beta$ -ocimene; (2) methyl chavicol; (3) methyl eugenol; (4) trans- $\beta$ -caryophyllene.

**Figure 8.1:** GC-MS chromatogram of 5% essential oil in hexane from *in vitro* leaves of *O. basilicum*.



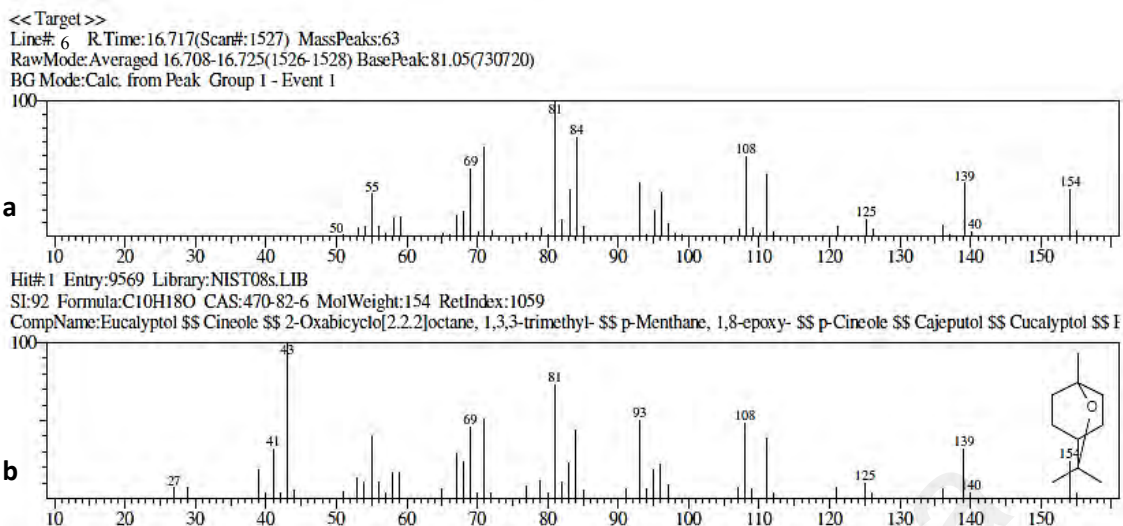
Main compounds: (1) 1,8-cineole; (2) trans- $\beta$ -ocimene; (3) camphor; (4) methyl chavicol.

**Figure 8.2:** GC-MS chromatogram of 5% essential oil in hexane from *ex vitro* leaves of *O. basilicum*.

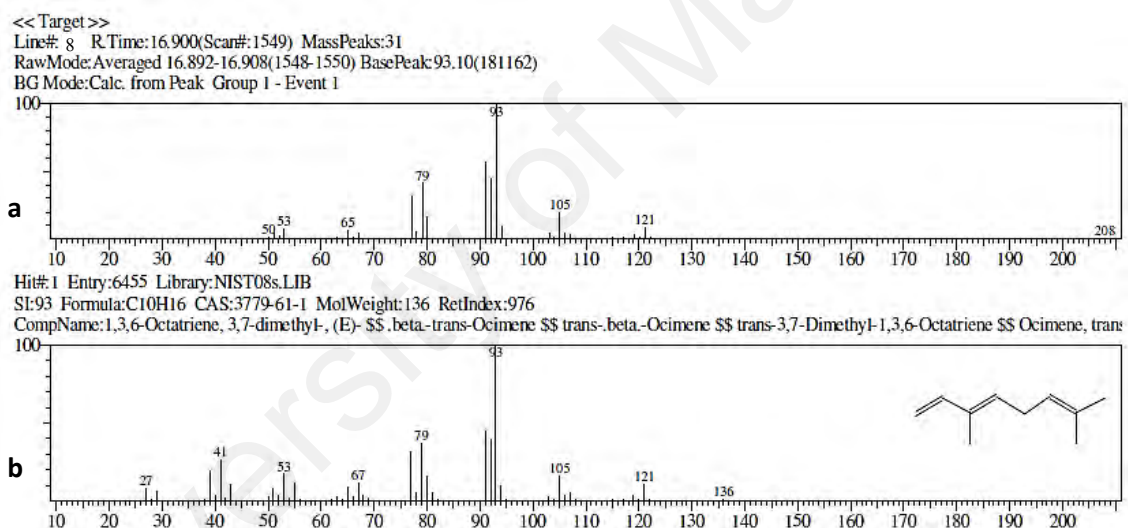


Main compounds: (1) 1,8-cineole; (2) trans- $\beta$ -ocimene; (3) camphor; (4) methyl chavicol.

**Figure 8.3:** GC-MS chromatogram of 5% essential oil in hexane from *in vivo* leaves of *O. basilicum*.

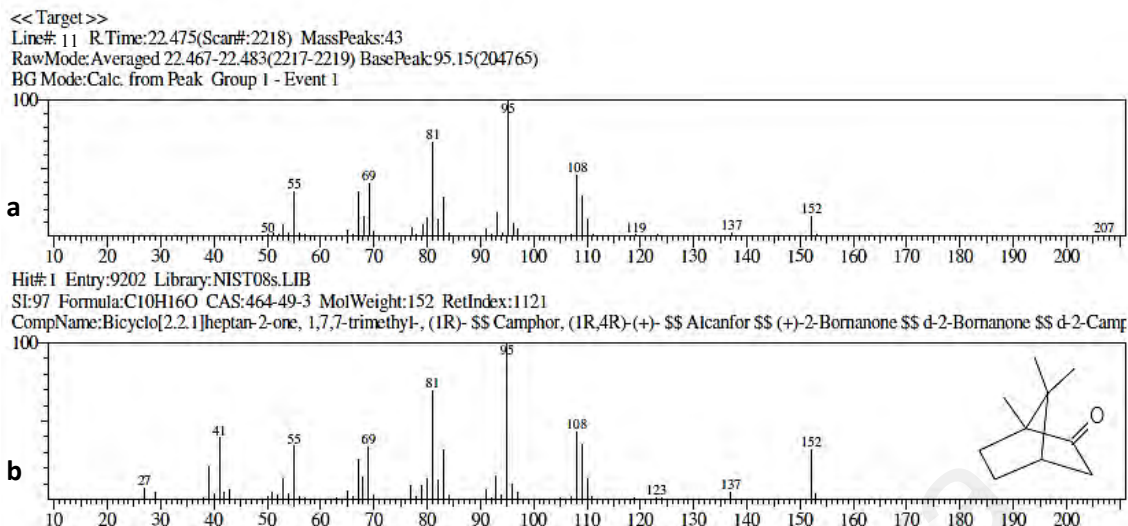


**Figure 8.4:** Mass spectrum of 1,8-cineole in the *ex vitro* leaves of *O. basilicum* (a), compared to in the NIST Mass Spectral Library (b).

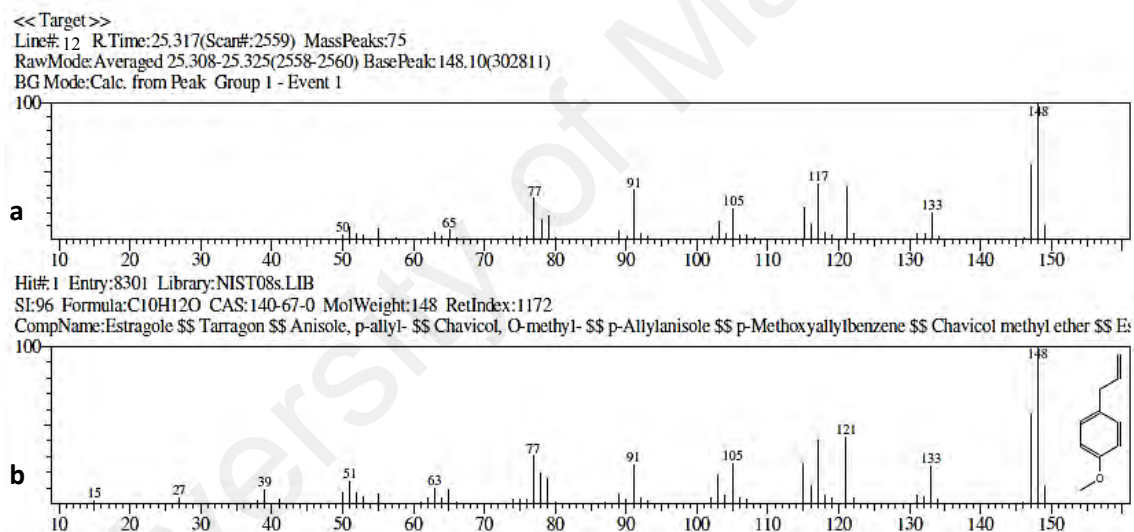


**Figure 8.5:** Mass spectrum of trans- $\beta$ -ocimene in the *ex vitro* leaves of *O. basilicum* (a), compared to in the NIST Mass Spectral Library (b).





**Figure 8.6:** Mass spectrum of camphor in the *ex vitro* leaves of *O. basilicum* (a), compared to in the NIST Mass Spectral Library (b).



**Figure 8.7:** Mass spectrum of methyl chavicol in the *ex vitro* leaves of *O. basilicum* (a), compared to in the NIST Mass Spectral Library (b).

#### 8.4 Summary of Results

1. The essential oil content and composition in leaves from the *in vitro*, *ex vitro* and *in vivo* grown plants of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol was determined for establishment of information on essential oil and compound accumulation of the plants at different developmental stages.
2. Essential oil content of the *in vitro* grown plants (1.99%) was lower than the *in vivo* grown plants (4.50%), but after 18 weeks of *ex vitro* acclimatization, the essential oil content increased to 2.38%.
3. Methyl chavicol was the main constituent found in the essential oils of *in vitro*, *ex vitro*, and *in vivo* plants. This finding confirmed that chemotype ‘methyl chavicol’ was used as the source of *O. basilicum* plant in this study.
4. The amount of methyl chavicol in the *in vitro* plants (93.71%) initially was higher than in the *in vivo* grown plants (66.29%), but after 18 weeks of *ex vitro* acclimatization, the amount dropped to 60.07%. The *ex vitro* and *in vivo* grown plants, which contained approximately the same methyl chavicol amount, were at almost the same developmental stage.

## CHAPTER 9: DISCUSSION

*O. basilicum*, or commonly known as ‘sweet basil’, is a multipurpose aromatic plant used in culinary, medicine, and perfumery, other than as an attractive, fragrant ornamental. In the present study, leaves and shoot tips of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol from two-month-old aseptic seedlings were used as initial explants for the induction of coloured callus formation, *in vitro* shoot multiplication, plant regeneration, and *in vitro* flowering through plant tissue culture technique. The *in vitro* regenerated plants were then analysed for their pollen and seed germinability, non-glandular and glandular trichome ultrastructures, and essential oil accumulation using pollen and seed germination test, SEM examination, and GC–MS analysis, respectively. The tissue culture techniques established in this study can be utilized to achieve mass propagation of this plant species, other than to solely rely on the conventional breeding method via the use of seeds.

The present study was divided into several experiments or chapters. These chapters included “coloured callus formation of *O. basilicum* L.”, “*in vitro* shoot multiplication and rooting of *O. basilicum* L.”, “*in vitro* flowering of *O. basilicum* L.”, “acclimatization and *ex vitro* flowering of *O. basilicum* L.”, “pollen and seed germination of *O. basilicum* L.”, “non-glandular and glandular trichome ultrastructure of *O. basilicum* L.”, and “essential oil accumulation in *O. basilicum* L.”.

Chapter 2 of this thesis described the induction of coloured callus formation from leaf and shoot tip explants of *O. basilicum* ‘Sweet Thai’, chemotype methyl chavicol. Exogenous supply of auxin and often in combination with cytokinin to culture media are essential for callus induction, but their requirement depends strongly on the

genotype and endogenous hormone content of the explant. Generally, an intermediate ratio of auxin and cytokinin promotes callusing (Ikeuchi *et al.*, 2013). In this experiment, different concentrations and combinations of PGRs were tested to find the optimum PGR that would best support induction of purple and light green callus formation, respectively, from leaf and shoot tip explants of this species.

The results presented in Table 2.1 and 2.2 showed that, both leaf and shoot tip explants of *O. basilicum* produced no callus when the explants were cultured on MS medium without PGR. However, when cultured on MS medium supplemented with combination of PGRs (BAP and NAA, BAP and 2,4-D), both leaf and shoot tip explants responded readily to all of the PGR combinations tested to produce callus, only differing in terms of the amount, colour, and texture of the callus produced. On MS medium supplemented with single PGR (BAP, GA<sub>3</sub>), none of the leaf and shoot tip explants showed callus formation.

Combination of cytokinin and auxin at low and equal concentrations was found to induce profuse callusing of *O. basilicum* leaf and shoot tip cultures with high pigment accumulation. The accumulation of pigments in leaf and shoot tip callus cultures was specific to type and concentration of PGRs. For example, in this study 0.5 mg l<sup>-1</sup> BAP combined with 0.5 mg l<sup>-1</sup> NAA and 1.0 mg l<sup>-1</sup> BAP combined with 0.5 mg l<sup>-1</sup> NAA worked best for purple-pigmented callus formation which was believed to contain anthocyanins, while 0.5 mg l<sup>-1</sup> BAP combined with 1.0 mg l<sup>-1</sup> 2,4-D and 1.0 mg l<sup>-1</sup> BAP combined with 1.0 mg l<sup>-1</sup> 2,4-D worked best for green-pigmented callus formation which was believed to contain chlorophyll.

The present study found that the highest percentage of purple (or anthocyanins) pigmentation and callus formation in both leaf and shoot tip cultures of *O. basilicum* appeared in MS medium supplemented with 0.5 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> NAA and also MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> NAA. In correspondence with the results in the present study, the high potency of combination of BAP and NAA in anthocyanin and callus formation was accomplished in *Crataegus sinaica* (Maharik *et al.*, 2009). BAP, in combination with NAA, not only induced the highest anthocyanin production, but also restored anthocyanin production through repeated subcultures and also slightly increased callus growth (Maharik *et al.*, 2009).

Based on the results of the present study, it was known that the rate of anthocyanin production was different dependent upon the type of auxins. Using NAA as auxin, combined with BAP, both leaf and shoot tip explants produced a higher amount of purple callus compared to using 2,4-D as auxin, combined with BAP. The success of anthocyanin and callus induction also depended on the plant species. While *O. basilicum* in the present study exhibited the highest production of purple callus using the combination of BAP and NAA, Sreenivas *et al.* (2011), in contrast, reported that *Bridelia stipularis* exhibited the highest accumulation of anthocyanins in the callus using the combination of BAP and 2,4-D, instead of the combination of BAP and NAA. The present study also found that cytokinin-auxin ratio influenced the anthocyanin and callus formation. Increasing the concentration of BAP to NAA reduced purple pigmentation as well as callus formation. A study by Abeda *et al.* (2014) however, reported that the highest increase in the anthocyanin content in *Hibiscus sabdariffa* L. callus treated with Kinetin and 2,4-D was accompanied with a low callus growth rate, which means the anthocyanin synthesis and callus growth are inversely correlated.

In the present study, while combination of BAP and NAA was found to be the best to facilitate purple callus formation in both leaf and shoot tip cultures of *O. basilicum*, combination of BAP and 2,4-D on the other hand was found to be the best to facilitate light green callus formation (MS medium supplemented with 0.5 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> 2,4-D and also MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> 2,4-D). Similarly, Khatak *et al.* (2014) proves outstanding role of 2,4-D as a stimuli to generate green callus from leaf explants of *Gymnema sylvestre* seedlings. Single 2,4-D generated green friable callus in almost 40 days which get shortened to 28 days when used in combination with kinetin; and combination of 2,4-D with NAA and BAP further lessen the time to initiate green callus to 20 days (Khatak *et al.*, 2014). The effectiveness of 2,4-D alone and in combination with cytokinin in inducing callus might be due to their role in DNA synthesis and mitosis (Sen *et al.*, 2014).

Based on the results of the present study, the highest percentage of light green callus formation from leaf and shoot tip explants of *O. basilicum* was obtained using 2,4-D as auxin, combined with BAP. In contrast, Siddique *et al.* (2006) obtained the highest percentage of green callus formation using NAA as auxin, whereby NAA combined with BAP induced green callus the highest in *Aristolochia indica*, whereas NAA combined with kinetin induced green callus the highest in *Hemidesmus indicus*. A report by Kim *et al.* (1992) showed that chlorophyll contents in the callus tissue of excised rice embryos increased with increasing concentration of kinetin to NAA, but the percentage of callus formation decreased. The present study however, showed a decrease both in green (or chlorophyll) pigmentation and overall callus formation with increasing concentration of BAP to 2,4-D. All these reports suggested that the type of auxins, the plant species, and the cytokinin-auxin ratio were the key factors in chlorophyll production and callus formation.

Chapter 3 of this thesis described the induction of *in vitro* shoot multiplication from leaf and shoot tip explants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol. Of the many factors that influence organogenesis *in vitro*, the most important single factor seemed to be the PGRs. In the current experiment, PGRs of different concentrations and combinations were tested to find the optimum PGR that would best support induction of shoot multiplication from leaf and shoot tip explants of this species.

The results in this study showed that, in all cultures initiated from leaf explants of *O. basilicum*, no direct organogenesis of shoots was observed on MS medium supplemented with and without PGRs. However, different results were obtained in shoot tip cultures of this species, where direct organogenesis was successfully achieved in all of the PGR treatments tested; but the number of shoots formed varied depending on the concentration and combination of PGRs. As demonstrated in Table 3.1, it was revealed that MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP was the best medium for the induction of shoot multiplication from shoot tip explants.

Shoot tip explants of *O. basilicum* cultured on PGR-free MS medium were unable to develop axillary shoots as the medium did not provide enough PGR for the shoot tips to break apical dominance, a phenomenon whereby the apical bud suppressed the growth of axillary buds. Thimann and Skoog (1934) demonstrated that the inhibition of axillary bud development was caused by the action of auxin diffusing out of the apical bud where it was produced, towards the base, causing the axillary buds to remain dormant. Application of specific exogenous PGRs, such as cytokinin, could break the apical dominance by counteracting the effect of endogenous auxin, thus inducing axillary bud development (Sachs and Thimann, 1967).

Several authors have reported the stimulating effect of cytokinins, either alone or in combination with other PGRs on axillary bud development from shoot tips of various species, including *Eurycoma longifolia* (Hussein *et al.*, 2005), *Exacum travancoricum* (Janarthanam and Sumathi, 2010), *Vernonia cinerea* (Maharajan *et al.*, 2010) and others. The results in the present study, which showed maximum shoot formation on MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP, was in agreement with these reports, suggesting cytokinin as an axillary bud induction stimulus.

The concentration of cytokinin influenced the number of shoots formed. In the present study, using BAP alone, the number of shoots formed per explant increased with an increase of BAP concentration up to 1.0 mg l<sup>-1</sup>. Further increase in BAP concentration did not improve, but reduced the number of shoots per explant. Begum *et al.* (2002) and Siddique and Anis (2008) obtained similar pattern of shoot multiplication in *O. basilicum* from shoot tip and nodal explants using various types of cytokinin applied singly, where shoot multiplication increased to the optimum concentration of cytokinin and then decreased above that optimum concentration. This shoot number reduction might be caused by the toxicity of BAP at higher concentrations. Asghari *et al.* (2012) reported that excess PGRs in culture medium was toxic and might lead to genetic, physiological, and morphological changes, resulting in a reduction of the multiplication rate *in vitro*.

The present study found that single BAP was more effective than combination of BAP and NAA for inducing axillary bud development from shoot tip explants of *O. basilicum*, as the addition of auxin to BAP induced callusing. This callus layer may have prevented uptake of the nutrients from the medium (Kielkowska and Havey, 2012), limiting shoot multiplication. In contrast, Daniel *et al.* (2010) reported a better



response of *O. basilicum* nodal explants in terms of axillary bud development when treated with BAP and auxins in combination as compared to treatment in BAP alone.

The present study showed that GA<sub>3</sub> alone stimulated increased shoot length in *O. basilicum*. The role of GA<sub>3</sub> in shoot elongation has been reported in other plant species like *Lens culinaris* Medik (Naeem *et al.*, 2004) and *Asparagus officinalis* (Saharan, 2010). However, the results of the present study showed that GA<sub>3</sub> alone gave only a mild effect on axillary bud development. In most cases, GA<sub>3</sub> did not appear to be essential for shoot multiplication (Gresshoff, 1978). Sahoo *et al.* (1997) reported that GA<sub>3</sub> alone, regardless of concentration, was unsuitable for shoot proliferation in *O. basilicum* as they yielded inferior shooting responses. Naeem *et al.* (2004) also reported a lack of branching in *L. culinaris* Medik plants treated with GA<sub>3</sub>.

Multiple shoots were cultured on half-strength MS basal medium to promote *in vitro* rooting. Table 3.2 demonstrated that 100% of the shoots developed roots on the medium. Like any other morphogenic process, rooting is an energy-consuming process and hence carbon source is desired. Sugars were reported to have an osmotic role and also act as a source of energy and carbon in inducing shoot and root formation. Hazarika (1999) reported that *in vitro* preconditioning of citrus microshoots with sucrose concentration of 3% was found optimum for subsequent *ex vitro* survival and growth. The present study found that the application of 3% sucrose was satisfactory for *in vitro* rooting of *O. basilicum* in order to obtain healthy roots and plantlets. Other than that, the concentration of MS salt used in the rooting medium was reduced to half. This is to modify culture-induced phenotype towards autotrophy in cultures that have persistent leaves that live longer and would be more photosynthetically productive *ex vitro* (Hazarika, 2003).

Chapter 4 of this thesis described the induction of *in vitro* flowering from leaf and shoot tip explants and multiple shoots of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol. *In vitro* flowering largely depends upon the levels and interaction of exo and endogenous phytohormones, sugars, minerals and phenolics (Murthy *et al.*, 2012). Different concentrations and combinations of PGRs were tested to find the optimum PGR that would best support induction of *in vitro* flowering from leaf and shoot tip explants, and to measure the efficacy of that optimum PGR to induce *in vitro* flowering from multiple shoots of this species.

For leaf explants of *O. basilicum*, neither direct nor indirect organogenesis was observed. Therefore, no flowers were formed when the explants were cultured on MS medium supplemented with and without PGRs. However, for shoot tip explants of this species, *in vitro* flowering was successfully achieved on MS medium supplemented with GA<sub>3</sub>. Multiple shoots also flowered on the GA<sub>3</sub>-containing MS medium. Higher percentage of *in vitro* flowering was obtained from the multiple shoots compared to from the shoot tip explants.

PGR requirements for *in vitro* flowering are variable depending on the plant species. *Bacopa chamaedryoides* (Haque and Ghosh, 2013) for example, flowered when treated with cytokinin alone, with BAP being more effective than kinetin. Other plant species like *Dioscorea zingiberensis* (Huang *et al.*, 2009), *Cleome viscosa* (Rathore *et al.*, 2013), and *Rosa hybrida* cv. Fairy Dance (Zeng *et al.*, 2013) required a combination of BAP and auxins to induce maximum *in vitro* flowering.

The results of the present study, however, showed that BAP alone and in combination with NAA was ineffective for the induction of *in vitro* flowering from the

shoot tip explants. Similar results was reported by Sudhakaran and Sivasankari (2002) that both BAP alone and BAP combined with NAA did not induce *in vitro* flowering from the nodal explants of *O. basilicum*; but they obtained *in vitro* flowering using a combination of BAP and IAA. The poor *in vitro* flowering observed may have been at least partially due to competition and/or nutritional deficiencies in the plant (Sivanesan and Jeong, 2007). In the present study, the presence of BAP which promoted vegetative growth may have resulted in the shoots formed to compete for nutrients from the medium and inhibited *in vitro* flowering.

In the present experiment, as demonstrated in Table 4.1 and 4.2, the highest percentage of explants with induced *in vitro* flowers was obtained on MS medium supplemented with  $1.0 \text{ mg l}^{-1}$  GA<sub>3</sub>, suggesting that GA<sub>3</sub> was essential for the induction of *in vitro* flowering in *O. basilicum*. Tang (2000), Ranasinghe *et al.* (2006), and Vásquez-Collantes *et al.* (2014) independently reported the stimulatory effect of single GA<sub>3</sub> on *in vitro* flowering from regenerated plantlets of *Panax ginseng*, *Gerbera jamesonii* Adlam, and “sundew” *Drosera capillaris* respectively. GA<sub>3</sub> was also demonstrated to promote early flowering and flower quality in acclimatized *Phalaenopsis* orchid (Cardoso *et al.*, 2012). On the other hand, Franklin *et al.* (2000) reported that GA<sub>3</sub> alone had no effect on flower bud induction in *Pisum sativum* but when combined, GA<sub>3</sub> enhanced the action of IBA and NAA to produce a higher number of *in vitro* flowers.

Gibberellin (GA) has been implicated in the control of flowering. MicroRNA156 (miR156)-targeted SQUAMOSA PROMOTER BINDING-LIKE (SPL) transcription factors promote flowering by activating miR172 and MADS box genes. When DELLA protein binds to SPL, the interaction between DELLA and SPL interferes with the SPL

transcriptional activity and consequently delays flowering through inactivating miR172 in leaf and MADS box genes at shoot apex under long-day conditions or through inactivating MADS box genes at shoot apex under short-day conditions (Yu *et al.*, 2012). GA accelerates flowering through degradation of DELLA proteins, hence promotes SPL transcriptional activity and activating miR172 and MADS box genes (Yu *et al.*, 2012).

The present study showed that, at GA<sub>3</sub> concentrations higher than 1.0 mg l<sup>-1</sup>, the percentage of *in vitro* flowering in *O. basilicum* decreased. Similar results was observed in *Panax ginseng*, which showed reduction in the percentage of *in vitro* flowering with increasing concentration of GA<sub>3</sub> above its optimum concentration (Tang, 2000). This flower reduction occurred may be due to the fact that these GA<sub>3</sub> concentrations may be above those required for the flowering of the species (Cardoso *et al.*, 2012). *O. basilicum* also flowered on PGR-free MS medium, similar to *Cucumis sativus* (Kielkowska and Havey, 2012), but the percentage of *in vitro* flowering on PGR-free MS medium was lower than on GA<sub>3</sub>-supplemented MS medium.

Based on the results of the present study, it was discovered that when induced on GA<sub>3</sub>-supplemented MS medium, multiple shoots produced a higher percentage of *in vitro* flowering compared to the shoot tip explants. The different percentages of *in vitro* flowering obtained from explants of different heights and ages cultured on the same medium probably trace to a different physiological status or stage of the explants (Zeng *et al.*, 2013), whereby the multiple shoots were taller and more mature than the shoot tips for transition from the vegetative to reproductive stage via GA<sub>3</sub> induction.

The occurrence of abnormal *in vitro* flowering in response to PGR treatments has been reported in a number of species. Zeng *et al.* (2013) observed many abnormalities among *in vitro* flowers of *Rosa hybrida* cv. Fairy Dance, especially in TDZ-supplemented MS medium, such as stems and leaves developing into flowers, flower bud forming on flowers, flower forming on other flowers, and flowers with fewer petals than normal and without stamens and pistils. In rose (hybrid tea) cv. “First Prize” (Vu *et al.*, 2006) and *Dendrobium* Sonia 17 (Tee *et al.*, 2008), *in vitro* flowers induced by cytokinins were smaller compared to *in vivo* flowers, with some flowers displayed malformations including incomplete floral structures and variation in shape, colour, and arrangement.

In the present study, normal *in vitro* flowers of *O. basilicum* exhibited complete floral structures with shapes, sizes, and colours similar to the *in vivo* flowers. However, some flowers induced on GA<sub>3</sub>-supplemented MS medium showed insignificant and temporary abnormalities of premature flowering. The flowers had smaller size, a lack of stamens and pistils, and sometimes white colour without purple pigmentation. Such abnormalities of the *in vitro* flowers occurred because the key regulatory genes for flowering and morphology in the plant have diverged and evolved to uniquely adapt to the environmental stress (Zeng *et al.*, 2013). The various abnormalities observed in *in vitro* flowers suggest that different conditions might be required for the induction and development of normal flowers (Tee *et al.*, 2008). In this study, for example, increasing the length and age of the explants used enhanced the production of normal flowers, as the multiple shoots produced a higher percentage of normal *in vitro* flowering than the shoot tips.

After a successful callus, shoot, root, and flower formation under *in vitro* conditions, *in vitro* regenerated plantlets were directed to acclimatization and flowering stages to complete the plant regeneration and reproduction processes under *ex vitro* conditions. Chapter 5 of this thesis described the promotion of acclimatization and *ex vitro* flowering from *in vitro* regenerated plants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol. Vermiculite followed by a soil mixture containing three parts garden soil to two parts potting mix were tested for their efficacy to promote acclimatization and *ex vitro* flowering of this species for adaptation and flowering of the plants under *ex vitro* conditions.

Transplantation stage is a major bottleneck in the micropropagation of many plant species. Plantlets or shoots that have grown *in vitro* have been exposed to a unique microenvironment under low level of light, aseptic conditions, on a medium containing ample sugar and nutrients to allow for heterotrophic growth, and in an atmosphere with high level of humidity, to provide minimal stress and optimum conditions for plant multiplication (Hazarika, 2003). These contribute to a culture-induced phenotype that cannot survive the environmental conditions when directly placed in a field. The typical physiological and anatomical characteristics of the micropropagated plantlets include reduced cuticular, epicuticular wax, and functional stomatal apparatus development; larger, circular, and of higher density stomata; and weak root system (Chandra *et al.*, 2010). Therefore, the micropropagated plantlets should be gradually acclimatized in order to ensure that sufficient number of plants survive and grow vigorously when transferred to the *ex vitro* environment of the greenhouse or field.

In the present study, rooted plantlets were transferred to moistened vermiculite and covered by transparent polythene bag in a growth chamber and later, the plantlets

were transplanted to garden soil and potting mix mixture in a field. As shown in Table 5.1, the whole acclimatization process in the present study resulted in an 80% plant survival rate. These acclimatization steps were conducted following the strategy that gradually introducing septic environment, increasing light intensity, reducing or completely eliminating sugar and nutrient contents for photoautotrophic growth, and lowering relative humidity could improve the internal and external structure of plantlets and give a more successful establishment in the field (Hazarika, 2003).

The *ex vitro* acclimatized plants of *O. basilicum* in the present study exhibited similar morphology with the *in vivo* grown plants. Both of them possessed green, elliptic leaves. During acclimatization to *ex vitro* conditions, leaf thickness generally increases, stomatal density decreases, and stomatal form changes from circular to elliptical one. Development of cuticle, epicuticular waxes, and effective stomatal regulation of transpiration occurs, leading to stabilization of water potential of field transferred plantlets (Chandra *et al.*, 2010). Ritchie *et al.* (1991) reported that leaves of chrysanthemum and sugar beet, which were initiated and developed at relative humidity below 100%, displayed increased epicuticular wax, improved stomatal functioning and reduced leaf dehydration.

After about 24 weeks of acclimatization, normal flowers developed from the *ex vitro* grown plants (Figure 5.3). The *ex vitro* flowers exhibited similar morphology with the normal *in vivo* flowers: they had corolla of 7 to 10 mm length, comprising of a white lower lip, a white with purple tinge 4-lobed upper lip, 1 pistil and 4 stamens. The soil might have provided sufficient nutrients for normal development of flowers *ex vitro*. The soil nourished with NPK fertilizer was reported to produce maximum flower yield in *Heliconia* sp. (Sushma *et al.*, 2012).

Chapter 6 of this thesis described the analysis of pollen and seed germination capability of *in vitro* regenerated plants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol. The germination rate of pollen grains and seeds was compared between the *in vitro*, *ex vitro*, and *in vivo* plants of this species for establishment of information on pollen and seed germinability of the plants at different developmental stages.

*In vitro* pollen germination was used in this experiment for the estimation of pollen viability as it determined the actual germination ability of pollen under suitable conditions (Tuinstra and Wedel, 2000; Lyra *et al.*, 2011). In the present study, 10% of sucrose was used in the pollen germination medium. This is due to the report by Lyra *et al.* (2011) that this concentration was the most suitable for rapid pollen germination in *Jatropha mollissima*, providing osmotic balance between the pollen grain and medium solutions, and providing energy for the tube growth.

The results presented in Table 6.1 showed that, *in vitro* grown plants did not show pollen germination on pollen germination medium. However, *ex vitro* grown plants exhibited pollen germination, with almost the same percentage of pollen germination with the *in vivo* grown plants. These results indicated that when tested on such pollen germination medium, the flowers developed under *in vitro* conditions produced non-germinable pollen but after the plants were acclimatized and transferred to the field, the flowers developed under *ex vitro* conditions produced germinable pollen, with more or less equal percentage of pollen germination with the *in vivo* flowers. Haque and Ghosh (2013) in contrast, reported good pollen viability with no significant difference in the percentage of pollen germination between the *in vitro*, *ex vitro*, and *in vivo* flowers of *Bacopa chamaedryoides*.



In the present study, pollen tubes of the germinated *O. basilicum* pollen grains showed bursting. The ruptured pollen tube ejected cytoplasm that coagulated into a mass resembling a pollen tube. This happened maybe because the pollen germination medium used was very poor in nutrients that it was insufficient to provide osmotic balance between the pollen grain and medium solution, and energy for the tube growth in this species. When the osmotic pressure of a medium is lower than that of the pollen, water moves into the pollen from the medium, resulting the cell wall of the pollen grain unable to sustain the additional pressure and burst (Adhikari and Campbell, 1998). Although the *ex vitro* and *in vivo* pollen grains showed abnormal pollen tube growth, it seems that only the germination mechanisms was affected, not the fertility of the pollen grains which was evident by the formation of normal seeds from the *ex vitro* and *in vivo* grown plants.

The results of the present study showed that, pollen grains of the *in vitro* flowers did not germinate; therefore no seed was set from the *in vitro* plant, in consistency with the results by Zhang and Leung (2000) in *Gentiana triflora* Pall. var. *axillariflora*. However, the *in vitro* regenerated plants later formed seeds under *ex vitro* conditions, and the *ex vitro* seeds exhibited similar morphology and germinability to the *in vivo* seeds. The absence of seeds from the *in vitro* plants could be caused by the lack of effective pollination, or the lack of viable pollen due to limited nutrients supplied by the flower induction medium during the peak of flowering stage where pollen maturation occurred (Jayaprakash and Sarla, 2001). Addition of  $\epsilon$ -amino caproic acid (EACA) to the pollen germination medium was reported to improve pollen germination percentage of *Cajanus cajan* (Jayaprakash and Sarla, 2001).

Chapter 7 of this thesis described the analysis of non-glandular and glandular trichome ultrastructure on leaves and flower petals of *in vitro* regenerated plants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol. The distribution, shape, and density of non-glandular and glandular trichomes on leaf and flower surfaces were compared between the *in vitro* and *in vivo* grown plants of this species for better understanding on the relationship between their morphology and the plant developmental stages.

In the present study, non-glandular and glandular trichomes were found on the surface of leaves and flower petals of *O. basilicum*. The function of these trichomes depends on their structure and position. Ultrastructural observations of the present study suggested that the pointed, hairy structure of non-glandular trichomes located on the leaf veins on the abaxial surface of leaves, at the tips of the abaxial surface of corollas, and at the bases of the adaxial surface of corolla tubes may serve as a mechanical barrier against various external factors, such as herbivores and pathogens, UV-B radiation, extreme temperatures, and excessive water loss by transpiration, as proposed by Werker (2000). On the other hand, the large spherical head structure of glandular trichomes (capitate and peltate) located over the surface on both the adaxial and abaxial surfaces of leaves and at the tips of the abaxial surface of corollas observed in the present study probably play a role in the secretion of essential oil or compounds to protect against predators and extreme environments, or to attract pollinators (Werker, 2000).

Generally, capitate glands consist of one to two stalk cells and one to two-celled head, rounded to pear shaped, while peltate glandular trichomes consist of a short stalk and a large flattened head of four to 18 cells, arranged in a disc or in two concentric circles (Ascensão *et al.*, 1995). The structure and size of capitate and peltate glandular

trichomes may vary in different species. In *O. basilicum* of the present study, capitate glandular trichomes were recognized by their small head of one to two cells, while peltate glandular trichomes were recognized by their large head of four cells (Figure 7.1b). In *Leonotis leonurus*, otherwise, capitate glandular trichomes had a four-celled glandular head, while peltate glandular trichomes had a large head with eight secretory cells (Ascensão *et al.*, 1995).

Ioannidis *et al.* (2002) demonstrated the positive correlation between the number and volume of glandular trichomes per leaf area and the total oil content, supporting the idea that glandular trichomes contributed to the essential oil production in *O. basilicum*. Werker *et al.* (1993) reported that both peltate and capitate glands of *O. basilicum* were stained with Sudan IV and Ruthenium Red, indicating that they contained both lipophilic and polysaccharidic substances; therefore, both peltate and capitate glands contributed to essential oil production. In later work, Gang *et al.* (2001) demonstrated that essential oil phenylpropenes, including methyl chavicol are synthesized and accumulate exclusively in peltate glandular trichomes. Assays for phenylalanine ammonia lyase, the enzyme that catalyzes the first step in the biosynthesis of all phenylpropenes, and for chavicol *O*-methyltransferase, the enzyme that catalyzes the last step in the formation of methyl chavicol, showed that only peltate glands and not capitate glands contained the enzymes coding for phenylpropanoid components of *O. basilicum* essential oil (Gang *et al.*, 2002).

Based on the ultrastructural examinations as presented in Table 7.1, it was revealed that *in vitro* and *in vivo* leaves of the same age and position had similar number of capitate glands and peltate glands per leaf area on the adaxial and abaxial surfaces, except for fewer capitate glands on the adaxial surface of *in vivo* leaves. However,

peltate glands on the *in vitro* leaves had more partially-filled oil sacs, as indicated by their creased and wrinkled folds, compared to the *in vivo* leaves which had more fully-filled oil sacs. Gang *et al.* (2001) reported that the partially-filled peltate glands of *O. basilicum* which appeared wrinkled and creased, were the characteristic of immature developing glands, which normally became expanded on maturity as the oil sacs filled with the essential oil constituents. Consequently, the creased and wrinkled folds of the peltate glands on the *in vitro* leaves might be indicative of their immaturity; so only little essential oil was produced, leaving the oil sacs partially-filled. The peltate glands of the *in vivo* leaves which had more fully-filled oil sacs, on the other hand, might be more mature; hence contained higher amounts of essential oil constituents. This can be seen by the distended and smooth structure of the peltate glands. Therefore, it can be suggested that *in vitro* leaves had more immature peltate glands compared to the *in vivo* leaves.

Chapter 8 of this thesis described the analysis of essential oil content and composition in leaves of *in vitro* regenerated plants of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol. The essential oil content and composition in leaves between the *in vitro*, *ex vitro*, and *in vivo* grown plants of this species was compared for establishment of information on essential oil and compound accumulation of the plants at different developmental stages.

The present study showed that, essential oil at a level of 4.50% was extracted from *in vivo* leaves of *O. basilicum*. This amount was higher than levels reported for 15 studied varieties of *O. basilicum* from Brazil and the USA reported by Vieira and Simon (2006), which ranged from 0.58% to 1.68%. While linalool was found as the dominant compound in three studied *O. basilicum* varieties in Poland (Wesołowska *et al.*, 2012),

the present study found methyl chavicol as the dominant compound in all three *in vitro*, *ex vitro* and *in vivo* *O. basilicum* leaves under study. These conflicting results showed that variations in the essential oil content and composition of *O. basilicum* across countries was influenced by environmental or climatic conditions of the regions (Wesołowska *et al.*, 2012), resulting in chemotypic variations within the species. A chemotype designation usually occurs because the plant is grown in a different area of the world or a distinct climate that alters the chemistry of the plants.

In *O. basilicum*, as observed for other Lamiaceae, such as *Mentha piperita* and *Salvia officinalis* (Amelunxen, 1964; Croteau *et al.*, 1981), synthesis and secretion of secretory materials into the subcuticular space in the peltate glandular trichomes start already in very young leaves. During the accumulation of secreted materials in the subcuticular space of the peltate glandular trichomes, changes occur in the content of some components of the essential oil. These results confirm those of Maffei *et al.* (1989) for *M. piperita* which showed that there is quantitative and qualitative variability in oil constituents of the glandular trichomes that are related with age of the glandular trichomes.

In the present study, as presented in Table 8.1, *in vitro* plants initially had lower essential oil content (1.99%) than *in vivo* plants (4.50%), and the level increased to 2.38% after 18 weeks of acclimatization under *ex vitro* conditions. Similarly, Verma *et al.* (2012) obtained the highest yield of essential oil at full bloom stage, where the plants almost reached maturity. The lower essential oil content of the *in vitro* plants compared to the *ex vitro* and *in vivo* plants could be a result of the younger developmental stage of the *in vitro* plants, which lacked fully developed glandular trichomes. Peltate glandular

trichomes of the *in vitro* leaves in the present study seemed to be partially-filled, resulted in reduced total essential oil content of the *in vitro* leaves.

Meanwhile, methyl chavicol content in the essential oil of *in vitro* plants (93.71%) initially was higher than in *in vivo* plants (66.29%), but the amount decreased to 60.07% after 18 weeks of *ex vitro* acclimatization. The higher amount of methyl chavicol in the essential oil of the *in vitro* plants than that of the *ex vitro* and *in vivo* plants indicated that methyl chavicol may be actively synthesized at the younger stage of development of the regenerated plants. Deschamps and Simon (2010) revealed that *O. basilicum* essential oil content and composition depended on plant developmental stage, whereby the accumulation of methyl chavicol decreased over time as leaves matured, a result of the decreasing chavicol *O*-methyltransferase (CVOMT) transcript expression levels with leaf age. The young developing tissues appeared to be the primary sites of essential oil phenylpropanes biosynthesis, as young leaves displayed much higher levels of CVOMT enzyme activity and methyl chavicol accumulation than more developed leaves (Deschamps and Simon, 2010).

The same goes for the camphor of *S. officinalis*. Avato *et al.* (2005) reported that the higher amount of camphor in the oil from the micropropagated *S. officinalis* than the field grown mother plants could be correlated to the younger stage of development of the regenerated plants.

It was also noted that *ex vitro* plants showed an intermediate level of essential oil content between the *in vitro* plants and *in vivo* plants, and nearly the same methyl chavicol content as the *in vivo* plants. These indicated that the developing *ex vitro* plants were positioned at an intermediate stage of plant development, ready for the transition

from the younger *in vitro* stage towards maturity, and thus had nearly the same essential oil content and methyl chavicol content as the more mature *in vivo* plants. This finding was in agreement with Holm *et al.* (1989), who found that the micropropagated peppermints had a higher concentration of menthol during the first year than the conventionally propagated plants, but in the second year, the concentration of menthol was about the same as the conventionally propagated plants.

It was reported that, depending on the plant species and the adopted regeneration protocol, the *in vitro* regenerated plants can produce either the same or different essential oil components as that of the wild plants (Bertoli *et al.*, 2004; Prins *et al.*, 2010). In the present study, the obtained results indicated that the proposed *in vitro* culture protocol of *O. basilicum* can be used for the production *O. basilicum* plants with essential oil of similar chemotype.

## CHAPTER 10: CONCLUSION

In the present study, efficient protocols for coloured callus formation, *in vitro* shoot multiplication, *in vitro* plant regeneration, and *in vitro* flowering from leaf and shoot tip explants of two-month-old *O. basilicum* 'Sweet Thai', chemotype methyl chavicol aseptic seedlings were developed successfully. PGRs and type of explants were demonstrated to be important factors influencing the growth and development of callus, multiple shoots, regenerated plantlets, and flowers in *in vitro* cultures. These protocols can be applied for mass production of coloured callus, plants, and flowers containing high levels of uniformity in the aspects of pollen and seed germinability, non-glandular and glandular trichome ultrastructure, and essential oil content and composition.

The present study found that MS medium supplemented with 0.5 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> NAA and also MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> NAA induced the highest percentage of purple callus formation from both leaf and shoot tip explants of *O. basilicum*, with 100% of the explants produced callus after 6 weeks of culture. Most of the calluses were profuse, compact, and purple-dominant. Meanwhile, MS medium supplemented with 0.5 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> 2,4-D and MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> 2,4-D was found to induce the highest percentage of light green callus formation from both leaf and shoot tip explants of *O. basilicum*, with 100% of the explants produced callus after 6 weeks of culture. Most of the calluses were profuse, semi-compact, and light green-dominant.

The results of the present study showed that MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP was the optimum medium that supports the best induction of shoot



multiplication from shoot tip explants of *O. basilicum*, with 100% of explants producing multiple shoots, having a mean of  $5.00 \pm 0.28$  shoots per explant averaging  $1.42 \pm 0.11$  cm in length after 8 weeks of culture. However, MS medium, with all of the PGR treatments tested, did not support induction of shoot multiplication from leaf explants. All (100%) multiple shoots showed root formation on half-strength MS medium, with mean root length of  $4.21 \pm 0.29$  cm after 4 weeks of subculture.

MS medium supplemented with  $1.0 \text{ mg l}^{-1}$  GA<sub>3</sub> was demonstrated to best support induction of *in vitro* flowering from shoot tip explants of *O. basilicum*, with 40% of explants producing *in vitro* flowers before the 20<sup>th</sup> week of culture. When multiple shoots were transferred to MS medium supplemented with  $1.0 \text{ mg l}^{-1}$  GA<sub>3</sub>, 100% of the shoots produced normal *in vitro* flowers with mean 14 flowers per shoot before the 16<sup>th</sup> week of culture. However, MS medium, with all of the PGR treatments tested, did not support induction of *in vitro* flowering from leaf explants.

The rooted plantlets were successfully established *ex vitro* with an 80% survival rate after 8 weeks of acclimatization in vermiculite followed by 10 weeks of growth in soil (3 parts garden soil to 2 parts potting mix), obtaining a mean height of  $25.43 \pm 2.59$  cm. The *ex vitro* acclimatized plants exhibited similar morphology to the *in vivo* mother plant and showed normal flower development after 24 weeks of acclimatization.

In *in vitro* pollen germination test, pollen grains of the *in vitro* plants did not germinate on the pollen germination medium, and no seed was formed from the *in vitro* plants. Pollen grains of the *ex vitro* plants, on the other hand, showed a 43.24% germination rate, which did not differ significantly to the *in vivo* plants (41.67% germination rate). These results indicated that *ex vitro* and *in vivo* pollen grains had

potential in developing seeds upon fertilization. Seeds derived from the *ex vitro* plants showed a 62.26% germination rate by seed germination test, which did not differ significantly to the *in vivo* plants (62.50% germination rate). *Ex vitro* plants displayed similar pollen and seed germinability as the *in vivo* mother plants.

Ultrastructural study showed that both *in vitro* and *in vivo* plants of *O. basilicum* possessed non-glandular and glandular trichomes on their leaf and flower surfaces. Non-glandular trichomes serve as protection against extreme environment, while glandular trichomes function in the secretion of essential oil. Leaves from both *in vitro* and *in vivo* plants bore non-glandular trichomes on the leaf veins of the abaxial surface and glandular trichomes over the adaxial and abaxial surfaces. Upper lip of corollas from both *in vitro* and *in vivo* plants bore non-glandular trichomes at the base of corollas on the adaxial surface, and at the tip of corollas on the abaxial surface. Upper lip of corollas from *in vitro* plants bore glandular trichomes at the tip of corollas on the abaxial surface only, but upper lip of corollas from *in vivo* plants bore glandular trichomes in the centre of corollas on the adaxial surface and at the tip of corollas on the abaxial surface. It was found that on both sides of leaves, there was no significant difference in the number of peltate and capitate glands per leaf area between the leaves of *in vitro* and *in vivo* plants, except for fewer capitate glands on the adaxial surface of *in vivo* leaves. However, most of the glands on the *in vitro* leaves and upper lip of corollas appeared creased and wrinkled, indicating that they were partially-filled with the essential oil constituents; whereas most of the glands on the *in vivo* leaves and upper lip of corollas appeared distended and smooth, indicating that they were fully-filled with the essential oil constituents.

Essential oil content of the *in vitro* plants (1.99%) was lower than the *in vivo* plants (4.50%), but after 18 weeks of acclimatization under *ex vitro* conditions, the essential oil content increased to 2.38%. GC-MS analysis revealed that all *in vitro*, *ex vitro*, and *in vivo* plants of *O. basilicum* contained methyl chavicol as their main essential oil constituent. However, the amount of methyl chavicol in the essential oil of the *in vitro* plants (93.71%) was higher than in the *in vivo* plants (66.29%), but the amount dropped to 60.07% after 18 weeks of acclimatization *ex vitro*.

Although the micropropagated plants under *in vitro* conditions showed some differences, such as flowers without pollen germination and seed formation, fewer fully-filled peltate glandular trichomes, lower essential oil content, and higher methyl chavicol content compared to the *in vivo* mother plants, after they were acclimatized to *ex vitro* conditions, the micropropagated plants eventually displayed similar leaf and flower morphology, pollen and seed germinability, essential oil content, and methyl chavicol content as the *in vivo* mother plants. These differences of the *in vitro* plants compared to the *in vivo* plants could be a result of a younger stage of development of the regenerated plants, whilst the *ex vitro* plants, which had nearly the same characteristics as the *in vivo* plants, were positioned at an intermediate stage of plant development, ready for the transition from the younger *in vitro* stage towards maturity.

Coloured callus formation, *in vitro* shoot multiplication, and *in vitro* flowering of *O. basilicum*, if defined precisely can provide ideal model system for the study of morphology, physiology, and biochemistry in *in vitro* regenerated plants at different developmental stages, besides for indoor ornamental purpose. The success of this study can be useful for mass production of plants and production of valuable secondary metabolites such as essential oil, pigments, and constituent compounds, which are

important for food and pharmaceutical industries, from this *O. basilicum* 'Sweet Thai', chemotype methyl chavicol from Malaysia.

In future, work should focus on the assessment of biological activities of *O. basilicum* extracts. Molecular work can be combined with tissue culture to improve quality of the regenerated plants.

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## LIST OF PUBLICATIONS AND PAPERS PRESENTED

### Articles published in academic journals

1. **Manan, A.A.**, Taha, R.M., Mubarak, E.E., & Elias, H. (2016). *In vitro* flowering, glandular trichomes ultrastructure, and essential oil accumulation in micropropagated *Ocimum basilicum* L. *In Vitro Cellular and Developmental Biology – Plant*. 52(3), 303-314.
2. Elias, H., Taha, R.M., Hasbullah, N.A., Mohamed, N., **Manan, A.A.**, Mahmad, N., & Mohajer, S. (2015). The effects of plant growth regulators on shoot formation, regeneration and coloured callus production in *Echinocereus cinerascens* in vitro. *Plant Cell, Tissue and Organ Culture*. 120(2), 729-739.

### Presentations in conferences

1. **Manan, A.A.** and Taha, R.M. (2013). The effects of various hormones on *in vitro* regeneration and *in vitro* flowering of *Ocimum basilicum* L. *5<sup>th</sup> Global Summit on Medicinal and Aromatic Plants (GOSMAP-5)*. December 8-12, 2013. Marriott Resort & Spa, Miri, Sarawak, Malaysia.

### Awards

1. Prof. Dr. Rosna Mat Taha, Dr. Sadegh Mohajer, Noraini Mahmad, Hashimah Elias, Sakinah Abdullah, Norlina Rawi, **Aziemah Abdul Manan**, & Arash Khorasani Esmaeili (2015). Transformation of an aquatic plant (*Nelumbo nucifera* Gaertn.) to terrestrial environment through MS double-layered medium. *International Conference and Exposition on Inventions by Institutions of Higher Learning (Pecipta'15)*. December 4-6, 2015. Kuala Lumpur Convention Centre, Kuala Lumpur, Malaysia. (*bronze medal*)
2. Prof. Dr. Rosna Mat Taha, Dr. Sadegh Mohajer, Normadiha Mohamed, **Aziemah Abdul Manan**, Noraini Mahmad, Norlina Rawi, & Sakinah Abdullah (2015). *In vitro* flowering of selected ornamental plants for breeding programmes and commercialization. *National Innovation and Invention Competition through Exhibition 2015 (iCompEx'15)*. March 24-26, 2015. Politeknik Sultan Abdul Halim Mu'adzam Shah, Kedah, Malaysia. (*silver medal*)



3. Prof. Dr. Rosna Mat Taha, Dr. Sadegh Mohajer, Arash Khorasani Esmaeili, Noraini Mahmad, Hashimah Elias, **Aziemah Abdul Manan**, & Sakinah Abdullah (2015). Mass propagation of novel forage crops (sainfoin and red clover) through synthetic seeds technology on MS doubled layered medium. *National Innovation and Invention Competition through Exhibition 2015 (iCompEx'15)*. March 24-26, 2015. Politeknik Sultan Abdul Halim Mu'adzam Shah, Kedah, Malaysia. (*gold medal*)
4. Prof. Dr. Rosna Mat Taha, Elnaiem Elaagib Mubarak, Dr. Sadegh Mohajer, Hashimah Elias, **Aziemah Abd. Manan**, Sakinah Abdullah, Noraini Mahmad, & Norlina Rawi (2014). Alternative and novel application of carbendazim (antifungal) as a growth regulator in callus and regeneration of *Eucalyptus camaldulensis* Dehnh. *International Engineering Invention & Innovation Exhibition (i-ENVEX) 2014*. April 11-13, 2014. Universiti Malaysia Perlis, Perlis, Malaysia. (*silver medal*)
5. Prof. Dr. Rosna Mat Taha, Sadegh Mohajer, Noraini Mahmad, Azani Saleh, Hashimah Elias, & **Aziemah Abdul Manan** (2013). Artificial seeds of sainfoin (*Onobrychis sativa* L.) – A novel forage crop for ruminants feed. *International Conference and Exposition on Invention of Institutions of Higher Learning (Pecipta'13)*. November 7-9, 2013. Kuala Lumpur Convention Centre, Kuala Lumpur, Malaysia. (*silver medal*)
6. Prof. Dr. Rosna Mat Taha, Dr. Jamilah Syafawati Yaacob, Azani Saleh, **Aziemah Abdul Manan**, Normadiha Mohamed, Noraini Mahmad, Norlina Rawi, & Umami Nur Ain Abdul Razak (2013). Floral scientific handicraft and indoor garden derived from *in vitro* techniques. *Bioinnovation Awards 2013*. October 21-23, 2013. Persada Johor International Convention Centre, Johor, Malaysia. (*bronze medal*)
7. Prof. Dr. Rosna Mat Taha, Dr. Jamilah Syafawati Yaacob, **Aziemah Abdul Manan**, Norlina Rawi, Sakinah Abdullah, Sharifah Nurashikin Wafa Syed Mohd Thalal Wafa, Normadiha Mohamed, & Hashimah Elias (2013). *In vitro* flowering for production of floral handicraft and mini indoor garden. *24<sup>th</sup> International Invention, Innovation & Technology Exhibition (ITEX'13)*. May 9-11, 2013. Kuala Lumpur Convention Centre, Kuala Lumpur, Malaysia. (*gold medal*)

## Patents

1. Rosna Mat Taha, Jamilah Syafawati Yaacob; **Aziemah Abdul Manan**; Norlina Rawi; Sakinah Abdullah; Sharifah Nurashikin Wafa Syed Mohd Thalal Wafa. *Method of in vitro flowering*. PI 2013700394 (2013).

## **Workshops**

1. Workshop on recombinant molecular biology techniques. October 29-30, 2012.  
University of Malaya, Kuala Lumpur, Malaysia.

University of Malaya

## APPENDICES

### Appendix I

Formulations of MS medium (Murashige & Skoog, 1962).

| Components  | Amount (mg l <sup>-1</sup> )   |
|---|--|
| <b>Macronutrients</b> <ul style="list-style-type: none"> <li>• Ammonium nitrate (NH<sub>4</sub>NO<sub>3</sub>)</li> <li>• Calcium chloride (CaCl<sub>2</sub> · 2H<sub>2</sub>O)</li> <li>• Magnesium sulphate (MgSO<sub>4</sub> · 7H<sub>2</sub>O)</li> <li>• Potassium phosphate (KH<sub>2</sub>PO<sub>4</sub>)</li> <li>• Potassium nitrate (KNO<sub>3</sub>)</li> </ul>  | 1,650<br>440<br>370<br>170<br>1,900                                  |
| <b>Micronutrients</b> <ul style="list-style-type: none"> <li>• Boric acid (H<sub>3</sub>BO<sub>3</sub>)</li> <li>• Cobalt chloride (CoCl<sub>2</sub> · 6H<sub>2</sub>O)</li> <li>• Cupric sulphate (CuSO<sub>4</sub> · 5H<sub>2</sub>O)</li> <li>• Ferrous sulphate (FeSO<sub>4</sub> · 7H<sub>2</sub>O)</li> <li>• Manganese sulphate (MnSO<sub>4</sub> · 4H<sub>2</sub>O)</li> <li>• Potassium iodide (KI)</li> <li>• Sodium molybdate (Na<sub>2</sub>MoO<sub>4</sub> · 2H<sub>2</sub>O)</li> <li>• Zinc sulphate (ZnSO<sub>4</sub> · 7H<sub>2</sub>O)</li> <li>• Na<sub>2</sub>EDTA · 2H<sub>2</sub>O</li> </ul> | 6.2<br>0.025<br>0.025<br>27.8<br>22.3<br>0.83<br>0.25<br>8.6<br>37.2 |
| <b>Vitamins and organics</b> <ul style="list-style-type: none"> <li>• Myo-Inositol</li> <li>• Niacin</li> <li>• Pyridoxine · HCl</li> <li>• Thiamine · HCl</li> <li>• Glycine</li> </ul>  | 100<br>0.5<br>0.5<br>0.1<br>2  |

## Appendix II

SPSS output

a) *In vitro* shoot multiplication

| Shoot multiplication               |    |                         |       |       |       |       |        |
|------------------------------------|----|-------------------------|-------|-------|-------|-------|--------|
| Treatment                          | N  | Subset for alpha = 0.05 |       |       |       |       |        |
|                                    |    | 1                       | 2     | 3     | 4     | 5     | 6      |
| Duncan <sup>a</sup> all BAP, 2,4-D | 30 | .0000                   |       |       |       |       |        |
| Control                            | 30 |                         | .2333 |       |       |       |        |
| 1.5 GA3                            | 30 |                         |       | .5000 |       |       |        |
| 2.0 GA3                            | 30 |                         |       | .5000 |       |       |        |
| 1.0 BAP, 1.0 NAA                   | 30 |                         |       | .6000 | .6000 |       |        |
| 0.5 GA3                            | 30 |                         |       | .6000 | .6000 |       |        |
| 1.0 GA3                            | 30 |                         |       | .6000 | .6000 |       |        |
| 0.5 BAP, 1.0 NAA                   | 30 |                         |       | .6000 | .6000 |       |        |
| 0.5 BAP, 0.5 NAA                   | 30 |                         |       |       | .7667 | .7667 |        |
| 1.0 BAP, 0.5 NAA                   | 30 |                         |       |       |       | .8333 | .8333  |
| 1.5 BAP, 1.0 NAA                   | 30 |                         |       |       |       | .8333 | .8333  |
| 2.0 BAP, 1.0 NAA                   | 30 |                         |       |       |       | .8667 | .8667  |
| 1.5 BAP, 0.5 NAA                   | 30 |                         |       |       |       | .9333 | .9333  |
| 2.0 BAP, 0.5 NAA                   | 30 |                         |       |       |       | .9667 | .9667  |
| 0.5 BAP                            | 30 |                         |       |       |       |       | 1.0000 |
| 1.0 BAP                            | 30 |                         |       |       |       |       | 1.0000 |
| 1.5 BAP                            | 30 |                         |       |       |       |       | 1.0000 |
| 2.0 BAP                            | 30 |                         |       |       |       |       | 1.0000 |
| Sig.                               |    |                         | 1.000 | .355  | .108  | .063  | .141   |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

| Duncan           |    |                        |       |       |       |       |       |       |       |
|------------------|----|------------------------|-------|-------|-------|-------|-------|-------|-------|
| No. of shoots    |    |                        |       |       |       |       |       |       |       |
| treatment        | N  | Subset for alpha = .05 |       |       |       |       |       |       |       |
|                  |    | 1                      | 2     | 3     | 4     | 5     | 6     | 7     | 8     |
| all BAP, 2,4-D   | 30 | 0                      |       |       |       |       |       |       |       |
| control          | 30 |                        | 1.300 |       |       |       |       |       |       |
| 0.5 GA3          | 30 |                        |       | 2.000 |       |       |       |       |       |
| 1.0 GA3          | 30 |                        |       | 2.067 |       |       |       |       |       |
| 1.5 GA3          | 30 |                        |       | 2.167 |       |       |       |       |       |
| 2.0 GA3          | 30 |                        |       | 2.167 |       |       |       |       |       |
| 0.5 BAP, 1.0 NAA | 30 |                        |       | 2.294 |       |       |       |       |       |
| 0.5 BAP, 0.5 NAA | 30 |                        |       | 2.333 |       |       |       |       |       |
| 1.0 BAP, 1.0 NAA | 30 |                        |       | 2.400 |       |       |       |       |       |
| 1.0 BAP, 0.5 NAA | 30 |                        |       | 2.600 | 2.600 |       |       |       |       |
| 1.5 BAP, 1.0 NAA | 30 |                        |       | 2.633 | 2.633 |       |       |       |       |
| 2.0 BAP, 1.0 NAA | 30 |                        |       | 2.667 | 2.667 |       |       |       |       |
| 1.5 BAP, 0.5 NAA | 30 |                        |       |       | 3.200 | 3.200 |       |       |       |
| 0.5 BAP          | 30 |                        |       |       |       | 3.600 | 3.600 |       |       |
| 2.0 BAP, 0.5 NAA | 30 |                        |       |       |       |       | 4.067 | 4.067 |       |
| 1.5 BAP          | 30 |                        |       |       |       |       |       | 4.500 | 4.500 |
| 2.0 BAP          | 30 |                        |       |       |       |       |       | 4.633 | 4.633 |
| 1.0 BAP          | 30 |                        |       |       |       |       |       |       | 5.000 |
| Sig.             |    |                        | 1.000 | .090  | .098  | .226  | .158  | .105  | .154  |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

b) *In vitro* flowering

**Normal flowering**

Duncan<sup>a</sup>

| treatment         | N  | Subset for alpha = 0.05 |      |      |      |       |
|-------------------|----|-------------------------|------|------|------|-------|
|                   |    | 1                       | 2    | 3    | 4    | 5     |
| 0                 | 30 | .000                    |      |      |      |       |
| 1                 | 30 | .033                    | .033 |      |      |       |
| control           | 30 |                         | .200 | .200 |      |       |
| 2.0 GA3           | 30 |                         | .200 | .200 |      |       |
| 0.5 GA3           | 30 |                         |      | .233 | .233 |       |
| 1.5 GA3           | 30 |                         |      | .233 | .233 |       |
| 1.0 GA3           | 30 |                         |      |      | .400 |       |
| regenerated shoot | 30 |                         |      |      |      | 1.000 |
| Sig.              |    | .713                    | .083 | .742 | .083 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

**Abnormal flowering**

Duncan<sup>a</sup>

| treatment | N  | Subset for alpha = 0.05 |
|-----------|----|-------------------------|
|           |    | 1                       |
| control   | 30 | .000                    |
| 1.0 GA3   | 30 | .100                    |
| 1.5 GA3   | 30 | .100                    |
| 2.0 GA3   | 30 | .100                    |
| 0.5 GA3   | 30 | .133                    |
| Sig.      |    | .106                    |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

c) Acclimatization

**Plants survive**

**Descriptive Statistics**

|                    | N         | Minimum   | Maximum   | Mean      |            | Std. Deviation |
|--------------------|-----------|-----------|-----------|-----------|------------|----------------|
|                    | Statistic | Statistic | Statistic | Statistic | Std. Error | Statistic      |
| survive            | 30        | .00       | 1.00      | .8000     | .07428     | .40684         |
| Valid N (listwise) | 30        |           |           |           |            |                |

**Plant heights**

**Descriptive Statistics**

|                    | N         | Minimum   | Maximum   | Mean      |            | Std. Deviation |
|--------------------|-----------|-----------|-----------|-----------|------------|----------------|
|                    | Statistic | Statistic | Statistic | Statistic | Std. Error | Statistic      |
| height             | 30        | .00       | 41.50     | 25.4333   | 2.58741    | 14.17181       |
| Valid N (listwise) | 30        |           |           |           |            |                |

d) *In vitro* pollen germination

**Pollen**

Duncan<sup>a,b</sup>

| plant    | N   | Subset for alpha = 0.05 |       |
|----------|-----|-------------------------|-------|
|          |     | 1                       | 2     |
| in vitro | 100 | .0000                   |       |
| in vivo  | 84  |                         | .4167 |
| ex vitro | 111 |                         | .4324 |
| Sig.     |     | 1.000                   | .786  |

Means for groups in homogeneous subsets are displayed.

- a. Uses Harmonic Mean Sample Size = 97.044.  
 b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

e) Seed germination

**Group Statistics**

|      | plant    | N   | Mean  | Std. Deviation | Std. Error Mean |
|------|----------|-----|-------|----------------|-----------------|
| seed | ex vitro | 106 | .6226 | .48703         | .04730          |
|      | in vivo  | 104 | .6250 | .48647         | .04770          |

**Independent Samples Test**

|      |                             | Levene's Test for Equality of Variances |      | t-test for Equality of Means |         |                 |                 |                       |   |        |
|------|-----------------------------|---|------|------------------------------|---------|-----------------|-----------------|-----------------------|---|--------|
|      |                             | F                                       | Sig. | t                            | df      | Sig. (2-tailed) | Mean Difference | Std. Error Difference | 95% Confidence Interval of the Difference |        |
|      |                             |   |      |                              |         |                 |                 |                       | Lower                                     | Upper  |
| seed | Equal variances assumed     | .005                                    | .944 | -.035                        | 208     | .972            | -.00236         | .06718                | -.13480                                   | .13008 |
|      | Equal variances not assumed |   |      | -.035                        | 207.933 | .972            | -.00236         | .06718                | -.13480                                   | .13008 |

f) Glandular trichomes

**Group Statistics**

|                | Plants   | N | Mean   | Std. Deviation | Std. Error Mean |
|----------------|----------|---|--------|----------------|-----------------|
| Adaxialpeltate | in vitro | 5 | 8.0000 | 1.73205        | .77460          |
|                | in vivo  | 5 | 8.4000 | 1.14018        | .50990          |

**Independent Samples Test**

|                |                             | Levene's Test for Equality of Variances |      | t-test for Equality of Means |       |                 |                 |                       |   |         |
|----------------|-----------------------------|---|------|------------------------------|-------|-----------------|-----------------|-----------------------|---|---------|
|                |                             | F                                       | Sig. | t                            | df    | Sig. (2-tailed) | Mean Difference | Std. Error Difference | 95% Confidence Interval of the Difference |         |
|                |                             |   |      |                              |       |                 |                 |                       | Lower                                     | Upper   |
| Adaxialpeltate | Equal variances assumed     | .334                                    | .579 | -.431                        | 8     | .678            | -.40000         | .92736                | -2.53850                                  | 1.73850 |
|                | Equal variances not assumed |   |      | -.431                        | 6.919 | .679            | -.40000         | .92736                | -2.59810                                  | 1.79810 |

### Group Statistics

|                 | Plants   | N | Mean    | Std. Deviation | Std. Error Mean |
|-----------------|----------|---|---------|----------------|-----------------|
| adaxialcapitate | in vitro | 5 | 19.6000 | 1.67332        | .74833          |
|                 | in vivo  | 5 | 15.8000 | 2.28035        | 1.01980         |

### Independent Samples Test

|                  |                             | Levene's Test for Equality of Variances |      | t-test for Equality of Means |       |                 |                 |                       |   |         |
|------------------|-----------------------------|---|------|------------------------------|-------|-----------------|-----------------|-----------------------|---|---------|
|                  |                             | F                                       | Sig. | t                            | df    | Sig. (2-tailed) | Mean Difference | Std. Error Difference | 95% Confidence Interval of the Difference |         |
|                  |                             |   |      |                              |       |                 |                 |                       | Lower                                     | Upper   |
| Adaxial capitate | Equal variances assumed     | .554                                    | .478 | 3.004                        | 8     | .017            | 3.80000         | 1.26491               | .88311                                    | 6.71689 |
|                  | Equal variances not assumed |   |      | 3.004                        | 7.339 | .019            | 3.80000         | 1.26491               | .83678                                    | 6.76322 |

### Group Statistics

|                | plants   | N | Mean   | Std. Deviation | Std. Error Mean |
|----------------|----------|---|--------|----------------|-----------------|
| abaxialpeltate | in vitro | 5 | 6.8000 | 1.09545        | .48990          |
|                | in vivo  | 5 | 6.2000 | .83666         | .37417          |

### Independent Samples Test

|                 |                             | Levene's Test for Equality of Variances |      | t-test for Equality of Means |       |                 |                 |                       |   |         |
|-----------------|-----------------------------|---|------|------------------------------|-------|-----------------|-----------------|-----------------------|---|---------|
|                 |                             | F                                       | Sig. | t                            | df    | Sig. (2-tailed) | Mean Difference | Std. Error Difference | 95% Confidence Interval of the Difference |         |
|                 |                             |   |      |                              |       |                 |                 |                       | Lower                                     | Upper   |
| Abaxial peltate | Equal variances assumed     | .043                                    | .840 | .973                         | 8     | .359            | .60000          | .61644                | -.82152                                   | 2.02152 |
|                 | Equal variances not assumed |   |      | .973                         | 7.482 | .361            | .60000          | .61644                | -.83884                                   | 2.03884 |

### Group Statistics

|                 | plants   | N | Mean    | Std. Deviation | Std. Error Mean |
|-----------------|----------|---|---------|----------------|-----------------|
| abaxialcapitate | in vitro | 5 | 20.0000 | 1.58114        | .70711          |
|                 | in vivo  | 5 | 17.6000 | 2.19089        | .97980          |

### Independent Samples Test

|                  |                             | Levene's Test for Equality of Variances |      | t-test for Equality of Means |       |                 |                 |                       |   |         |
|------------------|-----------------------------|---|------|------------------------------|-------|-----------------|-----------------|-----------------------|---|---------|
|                  |                             | F                                       | Sig. | t                            | df    | Sig. (2-tailed) | Mean Difference | Std. Error Difference | 95% Confidence Interval of the Difference |         |
|                  |                             |   |      |                              |       |                 |                 |                       | Lower                                     | Upper   |
| Abaxial capitate | Equal variances assumed     | 2.906                                   | .127 | 1.986                        | 8     | .082            | 2.40000         | 1.20830               | -.38636                                   | 5.18636 |
|                  | Equal variances not assumed |   |      | 1.986                        | 7.278 | .086            | 2.40000         | 1.20830               | -.43524                                   | 5.23524 |



## *In vitro* flowering, glandular trichomes ultrastructure, and essential oil accumulation in micropropagated *Ocimum basilicum* L.

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**Abstract** The aromatic and medicinal properties of *Ocimum basilicum* L. (sweet basil) are related to the unique essential oil chemistry in different cultivars. This study describes efficient micropropagation and *in vitro* flowering protocols from shoot tips and reveals information on seed germination capability, glandular trichomes ultrastructure, and essential oil content and composition at different plant developmental stages from micropropagated *O. basilicum* 'Sweet Thai', chemotype methyl chavicol. Shoot tips from 2-mo-old aseptic seedlings were induced to proliferate shoots on Murashige and Skoog (MS) medium supplemented with 6-benzyl-aminopurine (BAP) and gibberellic acid (GA<sub>3</sub>) either alone or in combination with  $\alpha$ -naphthalenacetic acid (NAA). Maximum shoot formation was achieved in MS medium supplemented with 1.0 mg L<sup>-1</sup> BAP. The micropropagated plants were successfully acclimatized *ex vitro* with an 80% survival rate. All of the micropropagated plants flowered *in vitro* on MS medium supplemented with 1.0 mg L<sup>-1</sup> GA<sub>3</sub>. Relative to the mother plant, *in vitro* plants flowered at a younger stage of plant development but showed a lack of seed formation, fewer fully filled peltate glandular trichomes, lower essential oil content, and higher methyl chavicol content. *Ex vitro* plants flowered at an intermediate stage of plant development and formed seed with nearly the same seed germinability, essential oil content, and methyl chavicol content as the mother plant.

**Keywords** *Ocimum basilicum* · 'Sweet Thai' basil · Micropropagation · *In vitro* flowering · Essential oil

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### Introduction

*Ocimum basilicum* L. (sweet basil) belongs to the family Lamiaceae and is a multipurpose herb characterized by its rich and aromatic essential oil content. This genus is primarily grown in warm temperate and tropical regions in Asia, Africa, and Central and South America as a culinary herb and an attractive, fragrant ornamental (Simon *et al.* 1999; Carović-Stanko *et al.* 2010). The aromatic leaves, flowers, and seeds are added to foods and beverages for flavor; extracted as active ingredient for use in perfumes, soaps, cosmetics, and dental products; and are included in traditional herbal medicines to treat fevers, headaches, kidney problems, gum ulcers, childbirths, rheumatoid arthritis, and menstrual irregularities (The Herb Society of America 2003). Besides these traditional medical uses, recent scientific studies have demonstrated potent antioxidant (Jayasinghe *et al.* 2003), antiviral (Chiang *et al.* 2005), and anti-proliferative activities (Manosroi *et al.* 2006) of compounds in *O. basilicum* essential oil and leaf extracts.

*O. basilicum* essential oil is a mixture of numerous compounds, mainly methyl chavicol (estragole), linalool, 1,8-cineole (eucalyptol), eugenol, and methyl cinnamate (Wesołowska *et al.* 2012; Said-Al Ahl *et al.* 2015). These compounds possess several biological activities. For example, methyl chavicol has antispasmodic (Coelho-de-Souza *et al.* 1997), antimicrobial (Friedman *et al.* 2002), and local anesthetic properties (Silva-Alves *et al.* 2013), linalool has anti-inflammatory properties (Peana *et al.* 2002), 1,8-cineole has vasorelaxant properties (Lahlou *et al.* 2002), and eugenol has antioxidant properties (Ogata *et al.* 2000). However, the composition of essential oil varies depending on the cultivar, and the taxonomy is complicated by the existence of chemotypes or chemical races that do not differ significantly in morphology (Simon *et al.* 1990; Carović-Stanko *et al.* 2010).



Lawrence (1988) classified four major chemotypes: methyl chavicol-rich, linalool-rich, methyl eugenol-rich, and methyl cinnamate-rich. The developmental stage of the plant also influences the yield and composition of *O. basilicum* essential oil (Deschamps and Simon 2010; Verma *et al.* 2012).

Micropropagation provides an effective means of rapid propagation and large-scale production of uniform plants of selected cultivars while maintaining their genotypes (Arikat *et al.* 2004). The micropropagated plants may serve as efficient plant models to study the accumulation of volatile compounds at different developmental stages. *In vitro* flowering can be a useful tool to study the flowering process, to accelerate breeding programs, and to optimize the commercial production of specific compounds from floral organs (Zeng *et al.* 2013). The application of tissue culture for the production of secondary metabolites is beneficial, as this method enables rapid production of secondary metabolites because of rapid growth of cultures *in vitro*, year-round production of secondary metabolites irrespective of seasonal and climatic conditions, and avoidance of collecting endangered wild species (Pierik 1987; Arikat *et al.* 2004).

Several authors have reported on the micropropagation and *in vitro* flowering of *O. basilicum* (Begum *et al.* 2002; Sudhakaran and Sivasankari 2002). However, the plant growth regulator (PGR) requirements for shoot proliferation and *in vitro* flowering vary in different cultivars and chemotypes. Studies about seed quality derived from the flowers of micropropagated *O. basilicum* have not yet been documented. Ultrastructural and chemical information regarding essential oil content and composition from micropropagated *O. basilicum* is also very limited. Therefore, the goal of this study was to identify the PGR or combination of PGRs that would best support induction of shoot proliferation and *in vitro* flowering from shoot tips of *O. basilicum* 'Sweet Thai', chemotype methyl chavicol, and to assess seed germinability, glandular trichomes ultrastructure, and essential oil content and composition at different developmental stages. This information may facilitate mass production of plants containing high essential oil content and uniform levels of constituent compounds.

## Materials and Methods

**Plant materials and culture conditions** *O. basilicum* L. 'Sweet Thai', chemotype methyl chavicol, was used as the reference mother plant. Its morphological and aromatic characteristics were as follows: herb; compact; purple, square stems; green, narrowly ovate, gland-dotted leaves; purple spikes; white with purple tinge, bilabiate with a lower lip and a four-lobed upper lip, funnel-shaped flowers; black, ovoid, mucilaginous when wet seeds; and sweet, anise-like, slightly spicy aroma (Paton 1992; Simon *et al.* 1999;

Raghavan 2006). Seeds were collected from a single mother plant in a village garden in Perak, Malaysia. The seeds were washed three times with distilled water, each time for 5 min, and then surface sterilized with 70% Clorox® (5.25% sodium hypochlorite, The Clorox Company, Oakland, CA) plus 0.1% Tween-20, again with 40% Clorox® (The Clorox Company), and then with 20% Clorox® (The Clorox Company), each time for 6 min, and then rinsed with sterile distilled water. The seeds were then treated with 70% ethanol for 1 min and rinsed five times with sterile distilled water. The sterilization procedures were conducted in a Microflow laminar flow cabinet (MDH Ltd., Andover, Hampshire, England). Finally, the surface sterilized seeds were placed aseptically on 45 mL MS medium (Murashige and Skoog 1962; Duchefa Biochemie B.V., Haarlem, The Netherlands) containing 3% sucrose (Duchefa Biochemie B.V.) and 0.8% agar technical (Oxoid Ltd., Basingstoke, Hampshire, England), at pH 5.8, which was autoclaved at 121°C and 103.8 kPa for 20 min. The seeds were germinated in 200-mL glass jars with plastic caps (Megalab Supplies, Petaling Jaya, Malaysia) and incubated in a growth chamber maintained at 25 ± 2°C under a 16-h photoperiod with an irradiance of 50 μmol m<sup>-2</sup> s<sup>-1</sup> provided by Philips Lifemax TL-D 18 W cool daylight fluorescent tubes (Royal Philips, Amsterdam, The Netherlands). The aseptic seedlings were used as source of explants 2 mo after germination.

**Shoot proliferation** Shoot tips approximately 0.7 cm in length were excised from healthy, 2-mo-old seedlings and cultured vertically in sterile 60-mL PS specimen containers (Labchem Sdn. Bhd., Petaling Jaya, Malaysia) containing 15 mL MS medium supplemented with PGRs: 6-benzylaminopurine (BAP; Sigma-Aldrich® Co., St. Louis, MO) and gibberellic acid (GA<sub>3</sub>; Sigma-Aldrich® Co.) at a concentration of 0.5, 1.0, 1.5, or 2.0 mg L<sup>-1</sup> either alone or in combination with α-naphthaleneacetic acid (NAA; Sigma-Aldrich® Co.) at a concentration of 0.5 or 1.0 mg L<sup>-1</sup>. BAP and NAA were sterilized by autoclaving, while GA<sub>3</sub> was sterilized by membrane filtration using Acrodisc® syringe filter with 0.2 μm Supor® membrane (Pall Corp., Ann Arbor, MI). PGR-free MS medium was used as a control. All cultures were incubated in a growth chamber using the same culture conditions as described above. Subculturing to fresh medium was performed at 60-d intervals. The experimental design was a randomized complete block (RCBD) with two replicates of 15 explants each. The percentage of explants showing shoot proliferation, number of shoots per explant, and shoot lengths was determined after 8 wk of culture.

***In vitro* rooting, acclimatization, and *ex vitro* flowering** Thirty micropropagated shoots, 3.5 to 4.0 cm in length and with four or more leaves, were excised individually and transferred from the proliferation medium to the rooting medium



containing 45 mL half-strength MS medium without PGR in 300-mL glass jars with plastic caps (Megalab Supplies). All cultures were incubated in a growth chamber using the same culture conditions as described above. The percentage of shoots showing root formation and root lengths was evaluated after 4 wk.

After 6 wk of culture on rooting medium, 30 healthy plantlets with well-developed roots were removed from the rooting medium and the roots were washed gently under running tap water to remove agar. The plantlets were transferred to plastic pots containing moistened vermiculite and covered with transparent polyethylene bags to maintain high humidity (80% to 90% relative humidity). Holes were made in the bags over time to allow gaseous exchange with environment. The plantlets were incubated in a growth chamber maintained at continuous  $25 \pm 2^\circ\text{C}$  under a 16-h photoperiod with an irradiance of  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  provided by Philips Lifemax TL-D 18 W cool daylight fluorescent tubes (Royal Philips). After 8 wk, the hardened plantlets were transplanted to polyethylene bags containing a mixture of garden soil and potting mix in a 3:2 w/w ratio and maintained under shade with a 12-h photoperiod provided by sunlight. The plants were watered once a day. A 15-15-15 nitrogen-phosphorus-potassium (N-P-K) fertilizer was applied once every 3 wk. The percentage of surviving plants and plant heights was evaluated after 18 wk. The acclimatized plants are referred to as the 'ex vitro plants' and any flowers developed from the acclimatized plants are called the 'ex vitro flowers'. The morphology of the ex vitro flowers was compared with the normal in vivo flowers which developed from intact mother plants.

**In vitro flowering** Two sets of experiments were carried out for the induction of in vitro flowering. In setup I, in vitro flowering was induced from shoot tips approximately 0.7 cm in length that were derived from 2-mo-old seedlings. The media compositions and culture conditions were the same as in the previous shoot proliferation experiment. The experiment was set up in an RCBD with two replicates of 15 explants each. The percentage of explants producing in vitro flowers was evaluated after 20 wk of culture.

In setup II, the experiment was carried out to test the effectiveness of the PGR that induced the maximum in vitro flowering from shoot tips as determined in setup I. Shoots approximately 3.0 cm in length were excised individually from 5-mo-old micropropagated shoots developed in the optimum proliferation medium (MS medium supplemented with  $1.0 \text{ mg L}^{-1}$  BAP) and transferred to the optimum flower induction medium (MS medium supplemented with  $1.0 \text{ mg L}^{-1}$  GA<sub>3</sub>). All cultures were incubated in a growth chamber maintained at  $25 \pm 2^\circ\text{C}$  under a 16-h photoperiod with an irradiance of  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  provided by Philips Lifemax TL-D 18 W cool daylight fluorescent tubes (Royal Philips). The experiment was set up in an RCBD with two replicates of 15 shoots

each. The percentage of shoots with in vitro flowers and number of flowers per shoot were evaluated after 16 wk of culture.

**Seed germination test** Mature seeds were placed on a layer of cotton wool, moistened with tap water in a Petri dish. All cultures were incubated in a dark chamber at  $25 \pm 2^\circ\text{C}$ . The percentage of seeds that germinated was determined after 21 d from a minimum of 100 seeds, chosen randomly from five plants each for in vitro, ex vitro, and in vivo plants. The in vivo seeds served as control.

**Glandular trichomes ultrastructural investigation** The upper lip of corollas during anthesis and the third pair of leaves ( $5 \text{ mm} \times 5 \text{ mm}$ ) from three 3-mo-old plants each for in vitro and in vivo plants were fixed in 3% glutaraldehyde in Sorenson's phosphate buffer, pH 7 (Bozzola 2007), for 1 h at room temperature, post-fixed in 4% aqueous osmium tetroxide overnight at  $5^\circ\text{C}$ , and dehydrated through an ethanol series (from 10% to 100% ethanol) and an ethanol-acetone series (from 3:1 to 1:1 ethanol/acetone v/v, then to 100% acetone). Each step had a duration of 15 min. The samples were then critical-point dried with carbon dioxide, mounted on stubs, and dried overnight in a vacuum desiccator before being coated with gold. The prepared samples were viewed in a scanning electron microscope (SEM) using JEOL JSM-7001F SEM (JEOL Ltd., Tokyo, Japan), focusing on the glandular trichomes on the adaxial and abaxial surfaces of the corollas and leaves. Five replicates (observation areas) of  $1 \text{ mm}^2$  per sample of three were chosen randomly to evaluate the glandular trichomes ultrastructure for each plant type.

**Analysis of essential oil composition** Essential oils were extracted from 150 g of fresh leaves, collected from a minimum of 20 plants each for in vitro, ex vitro, and in vivo plants, by a 4-h hydrodistillation using a Clevenger type apparatus with heat provided by a Favorit heating mantle (PLT Scientific Sdn. Bhd., Puchong, Malaysia). The oils obtained were weighed, and the yields were expressed relative to the dry mass of leaves. The oil extracts were stored in glass vials at  $-20^\circ\text{C}$  until analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis of essential oil was performed using a Shimadzu GC-2010 coupled with a Shimadzu GCMS-QP 2010 Plus (Shimadzu Corp., Kyoto, Japan) equipped with a flame ionization detector (FID). One microliter of sample containing 5% essential oil in hexane was injected into a gas chromatograph equipped with DB-5MS capillary column ( $30 \text{ m} \times 250 \mu\text{m}$ , film thickness  $0.25 \mu\text{m}$ ) using helium as the carrier gas, with a flow rate of  $1 \text{ mL min}^{-1}$ , injector temperature of  $250^\circ\text{C}$ , and a split injection ratio of 10:1. The oven temperature was initially  $40^\circ\text{C}$  for 2 min, with temperature increasing at a rate of  $3^\circ\text{C min}^{-1}$  until it reached  $140^\circ\text{C}$  for 2 min, after which the



rate of temperature increase was  $10^{\circ}\text{C min}^{-1}$  until it reached  $250^{\circ}\text{C}$ , where the temperature was held for 5 min.

The Kovats retention indices of compounds of the essential oils were determined based on the alkane series  $\text{C}_6\text{--C}_{23}$ . Identification of individual compounds was carried out by comparing their mass spectra with the mass spectra in NIST Mass Spectral Library (2008) and by the means of their retention indices, compared with those in the literature (Adams 2007; Wesolowska *et al.* 2012; Nurzyńska-Wierdak *et al.* 2013). Data were given as percent mass for each compound. *In vitro* micropropagated plants were assessed after 20 wk of culture, *ex vitro* plants were assessed 18 wk after transfer from culture medium to soil, and *in vivo* plants (mature mother plants) were evaluated under natural growing conditions.

**Statistical analysis** Data were analyzed using independent-samples Student's *t* test for two-group samples or one-way analysis of variance (ANOVA) with confidence intervals calculated *via* Duncan's multiple range test (DMRT) for more than two-group samples at  $P=0.05$ , using IBM SPSS statistics version 22 software (IBM Corp., Armonk, NY). Results were presented as mean  $\pm$  standard error (SE).

## Results

**Shoot proliferation** Shoot tips cultured on PGR-free MS medium regenerated complete plantlets with no axillary shoot formation, even after 8 wk of culture. On MS medium supplemented with different PGRs, shoot tips showed initial bud break within 10 to 12 d of culture (Fig. 1a), and later shoots formed from the axillary buds and apical buds (Fig. 1b). The number of shoots formed averaged between two and five shoots per explant, depending on the concentration and combination of PGRs used. Out of various media tested, MS medium supplemented with  $1.0\text{ mg L}^{-1}$  BAP was found to be the best for shoot formation, producing 100% shoot proliferation with  $5.00\pm 0.28$  shoots per explant averaging  $1.42\pm 0.11$  cm in length after 8 wk of culture (Table 1). Further increase in BAP concentration reduced the number of shoots per explant and resulted in stunted growth, but the differences were not significant. BAP combined with NAA also reduced the number of shoots per explant compared to BAP alone, as their combination induced callusing. For BAP and NAA combinations, when the concentration of NAA was increased from 0.5 to  $1.0\text{ mg L}^{-1}$ , the number of shoots per explant decreased. Increasing the concentration of BAP to NAA from 0.5 to  $2.0\text{ mg L}^{-1}$  minimized the reduction in the number of shoots through suppressed callusing, thus enhancing the proliferation percentage and number of shoots per explant.  $\text{GA}_3$ , especially at  $1.5\text{ mg L}^{-1}$ , stimulated shoot elongation up to  $2.73\pm 0.24$  cm after 8 wk of culture. However, its effect on shoot

proliferation was small, with an average of only two shoots per explant.

### *In vitro* rooting, acclimatization, and *ex vitro* flowering

Individual regenerated shoots showed 100% rooting with a root length of  $4.21\pm 0.29$  cm after 4 wk of culture on the rooting medium containing half-strength MS medium. The plantlets had good rooting systems for transplantation after 6 wk. Rooted plantlets were successfully hardened after 8 wk of growth in vermiculite (Fig. 1c) and subsequently established in a field under *ex vitro* condition (Fig. 1d). Eighty percent of plants survived after 18 wk of acclimatization obtaining a mean height of  $25.43\pm 2.59$  cm. The *ex vitro* plants were similar in morphology to the *in vivo* plants. After about 24 wk of acclimatization, normal flowers developed from the *ex vitro* plants (Fig. 1e). The *ex vitro* flowers were similar in morphology to the normal *in vivo* flowers.

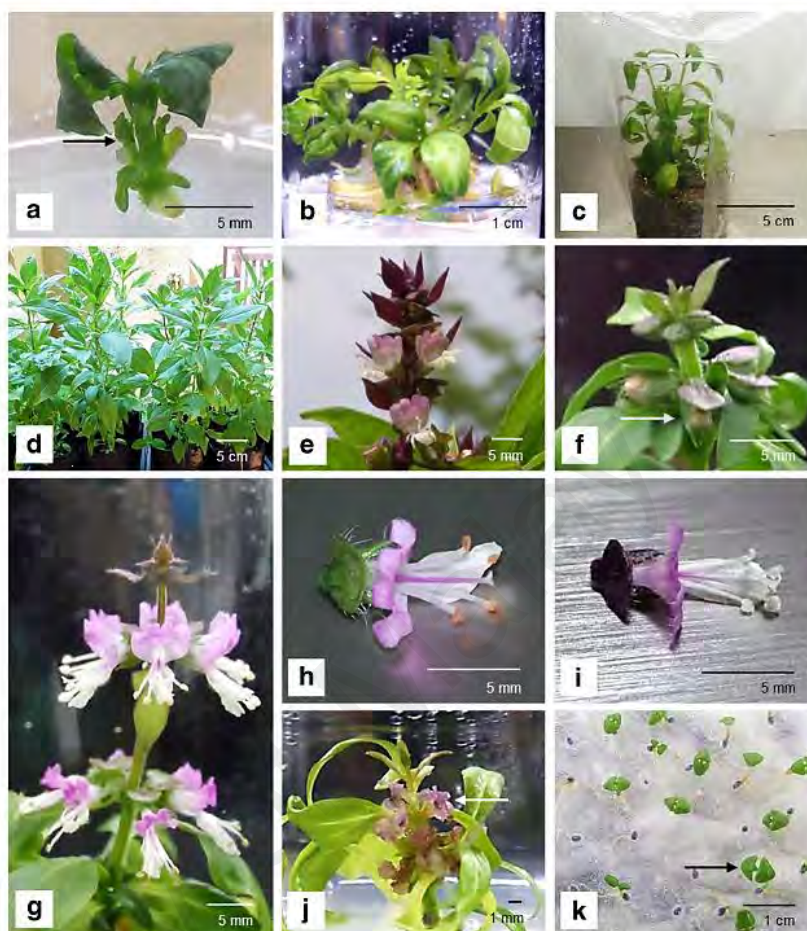
### *In vitro* flowering and morphological analysis of flowers

In setup I, *in vitro* flowering was induced from shoot tip explants. Flower buds were initiated (Fig. 1f), and later full bloom flowers formed from the developing shoots on the flower induction medium (Fig. 1g). Of the different media tested, the maximum percentage of *in vitro* flowering occurred in MS medium supplemented with  $1.0\text{ mg L}^{-1}$   $\text{GA}_3$ ; 40% of explants had normal flowers before the 20th wk of culture (Table 2). Normal flowers bloomed starting from the 6th wk of culture on the flower induction medium. The flowers were white and tinged with purple, and they were carried in spikes with six flowers per whorl. The *in vitro* flowers exhibited similar morphology compared to the *ex vitro* and *in vivo* flowers (Fig. 1h, i). A single flower lasted for about 1 to 2 wk *in vitro*. However, when treated with  $\text{GA}_3$ , 10 to 13% of the flowers were abnormal and premature, emerging as early as the 2nd wk of culture (Fig. 1j). These flowers lacked stamens and pistils and sometimes lacked purple pigmentation. These abnormalities were temporary and the plants produced normal flowers from the 6th through the 20th wk. The shoot tips cultured on PGR-free MS medium also developed flowers spontaneously, but the percentage of explants with normal flowers in this medium was lower than the  $\text{GA}_3$ -supplemented MS medium. BAP alone and in combination with NAA, at all concentrations tested, was ineffective for inducing *in vitro* flowering.

In setup II, *in vitro* flowering was induced from shoots micropropagated on MS medium supplemented with  $1.0\text{ mg L}^{-1}$  BAP. When transferred to MS medium supplemented with  $1.0\text{ mg L}^{-1}$   $\text{GA}_3$ , 100% of micropropagated shoots produced normal flowers, with a mean of 14 flowers per shoot, before the 16th wk of culture. The micropropagated shoots produced a significantly higher percentage of normal *in vitro* flowering as compared to the shoot tips. No abnormalities were detected in the flowers developed from the micropropagated shoots.



**Figure 1.** Micropropagation and *in vitro* flowering of *O. basilicum*. (a) Axillary buds (arrow) developed from a shoot tip after 12 d of culture in MS medium supplemented with  $1.0 \text{ mg L}^{-1}$  BAP; (b) micropropagated shoots formed after 8 wk of culture in MS medium supplemented with  $1.0 \text{ mg L}^{-1}$  BAP; (c) plantlets after 8 wk of hardening in vermiculite; (d) acclimatized plants established 10 wk after transfer to soil in the field; (e) *ex vitro* flowers developed after 24 wk of acclimatization in a mixture of three parts garden soil to two parts potting mix; (f) flower buds (arrow) initiated after 6 wk of culture in MS medium supplemented with  $1.0 \text{ mg L}^{-1}$   $\text{GA}_3$ ; (g) flowers in full bloom developed after 8 wk of culture in MS medium supplemented with  $1.0 \text{ mg L}^{-1}$   $\text{GA}_3$ ; (h) normal *in vitro* flower; (i) abnormal, premature *in vitro* flower (arrow); and (k) germinated seeds from an *ex vitro* plant with the first leaf pair (arrow).



**Seed germination test** No seed was formed on *in vitro* plants, but seeds were obtained from *ex vitro* and *in vivo* plants. Each *ex vitro* and *in vivo* flower produced four seeds per calyx and were visually similar. The seeds formed *ex vitro* and *in vivo* showed equivalent rates of germination, 62.26% and 62.50%, respectively (Table 3 and Fig. 1k).

**Glandular trichomes ultrastructural investigation** Ultrastructural SEM observations showed two types of glandular trichomes on the upper lip of the corollas and leaves: capitate glands and peltate glands. Capitate glands were composed of a base, a stalk, and a single or two-celled head, while peltate glands were composed of a base, a stalk, and a four-celled head (Fig. 2a).

There were similarities and differences in the structure and distribution of glandular trichomes on the *in vitro* and *in vivo* plants. On the upper lip of corollas, *in vitro* plants bore glandular trichomes on the abaxial surface only, but the *in vivo*

plants bore glandular trichomes on the adaxial and abaxial surfaces. On the adaxial surface, the glandular trichomes were scattered in the center of corollas, while on the abaxial surface, the glandular trichomes were concentrated at the tip of corollas near the corolla lobes. Most of the glandular trichomes on the *in vitro* flowers appeared creased and wrinkled (Fig. 2b).

For leaves, both the *in vitro* and *in vivo* plants bore glandular trichomes, scattered over the adaxial and abaxial surfaces. Except for fewer capitate glands on the adaxial surface of *in vivo* leaves, there was no other significant difference in the number of peltate and capitate glands per leaf area between the leaves of *in vitro* and *in vivo* plants (Table 3). However, the leaves from the *in vitro* plants contained fewer peltate glands with fully filled oil sacs compared to the *in vivo* plants (Fig. 2c, d). The fully filled oil sacs appeared distended and smooth, while the partially filled oil sacs appeared creased and wrinkled.



**Table 1.** Effects of different plant growth regulators on shoot proliferation after 8 wk of culture from shoot tips of *O. basilicum* cultured in MS medium with 3% sucrose and 0.8% agar

| Concentrations of plant growth regulators (mg L <sup>-1</sup> ) |         | % shoot proliferation | Mean number of shoots per explant | Mean shoot length (cm) |
|---|---------|-----------------------|-----------------------------------|------------------------|
| Cytokinin   | Auxin   |                       |                                   |                        |
| 0.0 BAP   | 0.0 NAA | 23.33±0.08 e          | 1.30±0.11 g                       | 1.83±0.12 b            |
| 0.5 BAP   | 0.0 NAA | 100.00±0.00 a         | 3.60±0.17 cd                      | 1.26±0.08 cd           |
| 1.0 BAP   | 0.0 NAA | 100.00±0.00 a         | 5.00±0.28 a                       | 1.42±0.11 c            |
| 1.5 BAP   | 0.0 NAA | 100.00±0.00 a         | 4.50±0.29 ab                      | 1.41±0.07 cd           |
| 2.0 BAP   | 0.0 NAA | 100.00±0.00 a         | 4.63±0.19 ab                      | 1.19±0.07 cd           |
| 0.5 BAP   | 0.5 NAA | 76.67±0.08 bc         | 2.33±0.19 f                       | 1.39±0.13 cd           |
| 1.0 BAP   | 0.5 NAA | 83.33±0.07 ab         | 2.60±0.18 ef                      | 1.17±0.10 cd           |
| 1.5 BAP   | 0.5 NAA | 93.33±0.05 ab         | 3.20±0.30 de                      | 1.20±0.09 cd           |
| 2.0 BAP   | 0.5 NAA | 96.67±0.03 ab         | 4.07±0.27 bc                      | 1.14±0.10 cd           |
| 0.5 BAP   | 1.0 NAA | 60.00±0.09 cd         | 2.29±0.29 f                       | 0.69±0.06 d            |
| 1.0 BAP   | 1.0 NAA | 60.00±0.09 cd         | 2.40±0.25 f                       | 0.97±0.12 d            |
| 1.5 BAP   | 1.0 NAA | 83.33±0.07 ab         | 2.63±0.21 ef                      | 1.21±0.10 cd           |
| 2.0 BAP   | 1.0 NAA | 86.67±0.06 ab         | 2.67±0.21 ef                      | 1.31±0.12 cd           |
| Gibberellin   | Auxin   |                       |                                   |                        |
| 0.5 GA <sub>3</sub>   | 0.0 NAA | 60.00±0.09 cd         | 2.00±0.19 f                       | 1.80±0.15 b            |
| 1.0 GA <sub>3</sub>   | 0.0 NAA | 60.00±0.09 cd         | 2.07±0.22 f                       | 2.16±0.20 b            |
| 1.5 GA <sub>3</sub>   | 0.0 NAA | 50.00±0.09 d          | 2.17±0.26 f                       | 2.73±0.24 a            |
| 2.0 GA <sub>3</sub>   | 0.0 NAA | 50.00±0.09 d          | 2.17±0.31 f                       | 2.16±0.20 b            |

Values followed by the same letter within columns are not significantly different at  $P=0.05$  as determined by DMRT

**Analysis of essential oil composition** The highest essential oil content, 4.50%, was found in the leaves of the *in vivo* plants. The essential oil content of the *in vitro* plants (1.99%) was lower than the *in vivo* plants, but after 18 wk of *ex vitro* acclimatization, it increased to 2.38% (Table 4).

Thirty main compounds were identified in the essential oils of *O. basilicum* leaves (Table 4). Methyl chavicol was the dominant compound in *in vitro*, *ex vitro*, and *in vivo* plants, although the relative quantity differed. The amount

of methyl chavicol in the *in vitro* plants (93.71%) initially was higher than in the *in vivo* plants (66.29%) but dropped to 60.07% after 18 wk of *ex vitro* acclimatization. Besides methyl chavicol, methyl eugenol (1.64%), trans- $\beta$ -ocimene (0.75%), and  $\beta$ -caryophyllene (0.60%) were found to present in high amounts in the *in vitro* plants, while trans- $\beta$ -ocimene (8.26–12.83%), 1,8-cineole (3.83–7.04%), and camphor (2.69–3.61%) were found in both the *ex vitro* and *in vivo* plants.

**Table 2.** Effects of different plant growth regulators on *in vitro* flowering from shoot tips and micropropagated shoots of *O. basilicum* cultured in MS medium with 3% sucrose and 0.8% agar

| Concentrations of plant growth regulators (mg L <sup>-1</sup> )                     |         | % normal <i>in vitro</i> flowering | % abnormal <i>in vitro</i> flowering |
|---|---------|------------------------------------|--------------------------------------|
| Setup I: <i>in vitro</i> flowering induced from shoot tips after 20 wk              |         |                                    |                                      |
| Gibberellin   | Auxin   |                                    |                                      |
| 0.0 GA <sub>3</sub>   | 0.0 NAA | 20.00±0.07 cd                      | 0.00±0.00 a                          |
| 0.5 GA <sub>3</sub>   | 0.0 NAA | 23.33±0.08 bc                      | 13.33±0.06 a                         |
| 1.0 GA <sub>3</sub>   | 0.0 NAA | 40.00±0.09 b                       | 10.00±0.06 a                         |
| 1.5 GA <sub>3</sub>   | 0.0 NAA | 23.33±0.08 bc                      | 10.00±0.06 a                         |
| 2.0 GA <sub>3</sub>   | 0.0 NAA | 20.00±0.07 cd                      | 10.00±0.06 a                         |
| Setup II: <i>in vitro</i> flowering induced from micropropagated shoots after 16 wk |         |                                    |                                      |
| Concentrations of plant growth regulators (mg L <sup>-1</sup> )                     |         | % normal <i>in vitro</i> flowering | % abnormal <i>in vitro</i> flowering |
| Gibberellin   | Auxin   |                                    |                                      |
| 1.0 GA <sub>3</sub>   | 0.0 NAA | 100.00±0.00 a                      | 0.00±0.00 a                          |

Values followed by the same letter within columns are not significantly different at  $P=0.05$  as determined by DMRT

**Table 3.** Comparison between the *in vitro*, *ex vitro*, and *in vivo* plants of *O. basilicum*

|   | <i>In vitro</i> plants | <i>Ex vitro</i> plants | <i>In vivo</i> plants |
|---|------------------------|------------------------|-----------------------|
| % seed germination  | No seed formed         | 62.26±0.05 a           | 62.50±0.05 a          |
| Mean number of peltate glands on 1 mm <sup>2</sup> of leaf (adaxial surface)  | 8.00±0.77 a            | —                      | 8.40±0.51 a           |
| Mean number of peltate glands on 1 mm <sup>2</sup> of leaf (abaxial surface)  | 6.80±0.49 a            | —                      | 6.20±0.37 a           |
| Mean number of capitate glands on 1 mm <sup>2</sup> of leaf (adaxial surface) | 19.60±0.75 a           | —                      | 15.80±1.02 b          |
| Mean number of capitate glands on 1 mm <sup>2</sup> of leaf (abaxial surface) | 20.00±0.71 a           | —                      | 17.60±0.98 a          |

Values followed by the same letter within rows are not significantly different at  $P=0.05$  as determined by Student's *t* test

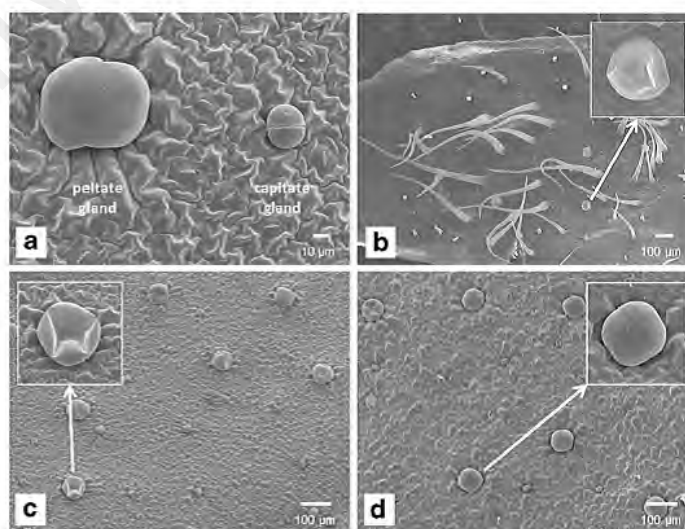
## Discussion

In the present study, shoot tips of *O. basilicum* from 2-mo-old aseptic seedlings were used as explants for micropropagation. Shoot tips cultured on PGR-free MS medium were unable to develop axillary shoots as the medium did not provide enough PGR for the shoot tips to break apical dominance. Thimann and Skoog (1934) demonstrated that the inhibition of axillary bud development was caused by auxin diffusing out of the apical bud where it was produced, causing the axillary buds to remain dormant. Application of specific exogenous PGRs, such as cytokinin, could break the apical dominance by counteracting the effect of endogenous auxin, thus inducing axillary bud development (Sachs and Thimann 1967). Several authors have reported the stimulating effect of cytokinins, either alone or in combination with other PGRs, on axillary bud development from shoot tips of various species, including *Eurycoma longifolia* (Hussein *et al.* 2005), *Exacum travancoricum* (Janarthanam and Sumathi 2010), and

*Vernonia cinerea* (Maharajan *et al.* 2010). The results of the present study, which showed maximum shoot formation on MS medium supplemented with 1.0 mg L<sup>-1</sup> BAP, were in agreement with these reports.

The concentration of cytokinin influenced the number of shoots that were formed. In the present study, using BAP alone, the number of shoots per explant increased with an increase of BAP concentration up to 1.0 mg L<sup>-1</sup>. Further increase in BAP concentration reduced the number of shoots per explant. Begum *et al.* (2002) and Siddique and Anis (2008) obtained similar results in *O. basilicum* from shoot tip and nodal explants. Shoot number reduction might be caused by the toxicity of BAP at higher concentrations. Narayanaswamy (1977) and Asghari *et al.* (2012) reported that excess PGRs were toxic and might lead to genetic, physiological, and morphological changes, resulting in a reduction of the proliferation rate *in vitro*. The present study found that BAP alone was more effective than BAP and NAA in combination for inducing axillary bud development, as the addition of auxin to BAP

**Figure 2.** Scanning electron micrographs of *O. basilicum*. (a) Glandular trichomes divided into two-celled head capitate gland and four-celled head peltate gland (bar = 10 μm); (b) distribution of glandular trichomes (arrow) on the abaxial surface of the upper lip of the corolla from an *in vitro* flower, showing their concentration at the tip of the flower petal (bar = 100 μm); (c) distribution of glandular trichomes on the abaxial surface of an *in vitro* leaf (bar = 100 μm); and (d) distribution of glandular trichomes on the abaxial surface of an *in vivo* leaf (bar = 100 μm). Note that the *in vitro* leaf had fewer fully filled peltate glands than the *in vivo* leaf.





**Table 4.** Essential oil content and composition (%) in *O. basilicum* leaves from *in vitro*, *ex vitro*, and *in vivo* plants

|  |      | <i>In vitro</i> plants <sup>z</sup> | <i>Ex vitro</i> plants <sup>y</sup> | <i>In vivo</i> plants <sup>x</sup> |
|--|------|-------------------------------------|-------------------------------------|------------------------------------|
| Essential oil content (%) (oil mass/leaf dry mass) |      | 1.99                                | 2.38                                | 4.50                               |
| Compounds (%)                                      | KI   |                                     |                                     |                                    |
| $\alpha$ -Pinene                                   | 942  | 0.05                                | 0.39                                | 0.36                               |
| Sabinene   | 973  | 0.11                                | 0.28                                | 0.23                               |
| $\beta$ -Pinene                                    | 977  | 0.03                                | 0.60                                | 0.38                               |
| $\beta$ -Myrcene                                   | 988  | 0.04                                | 1.89                                | 1.36                               |
| Limonene   | 1024 | 0.05                                | 0.45                                | 0.16                               |
| 1,8-Cineole  | 1027 | 0.27                                | 3.83                                | 7.04                               |
| <i>cis</i> - $\beta$ -Ocimene                      | 1031 | 0.02                                | 0.82                                | 0.54                               |
| <i>trans</i> - $\beta$ -Ocimene                    | 1045 | 0.75                                | 8.26                                | 12.83                              |
| Terpinolene  | 1080 | 0.07                                | 0.65                                | 0.53                               |
| $\beta$ -Linalool                                  | 1096 | 0.11                                | 0.73                                | 0.80                               |
| Camphor  | 1141 | 0.28                                | 2.69                                | 3.61                               |
| Methyl chavicol                                    | 1196 | 93.71                               | 60.07                               | 66.29                              |
| Bomyl acetate                                      | 1287 | 0.21                                | 0.74                                | 0.17                               |
| Elixene  | 1332 | 0.01                                | 0.54                                | tr                                 |
| $\beta$ -Elemene                                   | 1391 | 0.23                                | 1.78                                | 0.02                               |
| Methyl eugenol                                     | 1407 | 1.64                                | 2.50                                | 0.52                               |
| <i>trans</i> - $\beta$ -Caryophyllene              | 1425 | 0.60                                | 1.84                                | 0.49                               |
| <i>trans</i> - $\alpha$ -Bergamotene               | 1440 | 0.10                                | 1.50                                | 0.77                               |
| $\alpha$ -Guaiene                                  | 1442 | 0.08                                | 0.34                                | 1.07                               |
| <i>cis</i> - $\beta$ -Farnesene                    | 1461 | 0.34                                | 0.90                                | 0.51                               |
| $\alpha$ -Humulene                                 | 1464 | 0.17                                | 1.36                                | 0.22                               |
| <i>cis</i> -Muurolo-4(14),5-diene                  | 1469 | 0.02                                | 0.51                                | 0.06                               |
| Gemacrene D  | 1486 | 0.05                                | 0.70                                | 0.08                               |
| Bicyclogemacrene                                   | 1497 | 0.11                                | 0.82                                | 0.12                               |
| $\alpha$ -Bulnesene                                | 1503 | 0.14                                | 0.67                                | 0.17                               |
| $\gamma$ -Cadinene                                 | 1516 | 0.15                                | 1.22                                | 0.46                               |
| 1,10-di- <i>epi</i> -Cubenol                       | 1611 | 0.06                                | 0.65                                | 0.26                               |
| $\tau$ -Cadinol                                    | 1646 | 0.52                                | 2.25                                | 0.54                               |
| $\beta$ -Eudesmol                                  | 1660 | 0.06                                | 0.61                                | 0.28                               |
| $\alpha$ -Bisabolol                                | 1692 | 0.02                                | 0.09                                | 0.13                               |
| Others   |      | 0.00                                | 0.32                                | 0.00                               |
| Total  |      | 100.00                              | 100.00                              | 100.00                             |

KI Kovats index, relative to *n*-alkane series calculated on a DB-5MS column

<sup>z</sup> Micropropagated plants (*in vitro*), after 20 wk of culture

<sup>y</sup> Acclimatized micropropagated plants (*ex vitro*), 18 wk after transfer to soil

<sup>x</sup> Mature mother plants (*in vivo*), prior to flowering

induced callusing. This callus layer may have prevented uptake of the nutrients from the medium (Kielkowska and Havey 2012), limiting shoot proliferation. In contrast, Daniel *et al.* (2010) reported a better response of *O. basilicum* nodal explants in terms of axillary bud development when treated with BAP and auxins in combination as compared to treatment with BAP alone.

The present study showed that GA<sub>3</sub> stimulated increased shoot length in *O. basilicum*. A role for GA<sub>3</sub> in shoot elongation has been reported in other plant species like Pinto bean

(*Phaseolus vulgaris*, Marth *et al.* 1956), *Lens culinaris* Medik (Naeem *et al.* 2004), and *Asparagus officinalis* (Saharan 2010). However, GA<sub>3</sub> was not normally used for shoot multiplication. Sahoo *et al.* (1997) reported that GA<sub>3</sub> alone, regardless of concentration, was unsuitable for shoot proliferation in *O. basilicum* as it yielded inferior shooting responses. Naeem *et al.* (2004) also reported a lack of branching in *L. culinaris* Medik plants treated with GA<sub>3</sub>.

PGR requirements for *in vitro* flowering are variable depending on the plant species. *Bacopa chamaedryoides* (Haque



and Ghosh 2013) flowered when treated with cytokinin alone, with BAP being more effective than kinetin. Other plant species like *Dioscorea zingiberensis* (Huang *et al.* 2009), *Cleome viscosa* (Rathore *et al.* 2013), and *Rosa hybrida* cv. Fairy Dance (Zeng *et al.* 2013) required a combination of BAP and auxins to induce maximum *in vitro* flowering. The results of the present study, however, showed that BAP alone and in combination with NAA was ineffective for the induction of *in vitro* flowering from the shoot tip explants. Similar results were reported by Sudhakaran and Sivasankari (2002), although they induced *in vitro* flowering using a combination of BAP and IAA. The poor *in vitro* flowering observed may have been at least partially due to competition and/or nutritional deficiencies in the plant, perhaps induced by BAP, which promoted vegetative growth (Sivanesan and Jeong 2007).

In the present experiment, the maximum percentage of explants with *in vitro* flowers was obtained on MS medium supplemented with  $1.0 \text{ mg L}^{-1}$  GA<sub>3</sub>, suggesting that GA<sub>3</sub> was essential for the induction of *in vitro* flowering. Tang (2000), Ranasinghe *et al.* (2006), and Vásquez-Collantes *et al.* (2014) independently reported the stimulatory effect of GA<sub>3</sub> on *in vitro* flowering from regenerated plantlets of *Panax ginseng*, *Gerbera jamesonii* Adlam, and *Drosera capillaris*. GA<sub>3</sub> also promoted early flowering and flower quality in acclimatized *Phalaenopsis* orchid (Cardoso *et al.* 2012). On the other hand, Franklin *et al.* (2000) reported that GA<sub>3</sub> alone had no effect on flower bud induction in *Pisum sativum* but when combined with IBA and NAA, a higher number of *in vitro* flowers was produced.

Gibberellin (GA) has been implicated in the control of flowering. GA accelerates flowering through degradation of DELLA proteins that, when they bind to miR156-targeted SQUAMOSA PROMOTER BINDING-LIKE (SPL) transcription factors, interfere with SPL transcriptional activity and consequently delay flowering (Yu *et al.* 2012). *O. basilicum* also flowered on PGR-free MS medium, similar to *Cucumis sativus* (Kielkowska and Havey 2012), but the percentage of *in vitro* flowering on PGR-free MS medium was lower than on GA<sub>3</sub>-supplemented MS medium. The present study showed that, when induced on GA<sub>3</sub>-supplemented MS medium, micropropagated shoots produced a higher percentage of *in vitro* flowering compared to the shoot tips. The different percentages of *in vitro* flowering obtained from explants of different heights and ages cultured on the same medium probably trace to a different physiological status or stage of the explants (Zeng *et al.* 2013), whereby the micropropagated shoots were taller and more mature than the shoot tips for transition from the vegetative to reproductive stage via GA<sub>3</sub> induction.

The occurrence of abnormal *in vitro* flowering in response to PGR treatments has been reported. Zeng *et al.* (2013) observed many abnormalities among *in vitro* flowers of *R.*

*hybrida* cv. Fairy Dance, especially in TDZ-supplemented MS medium, such as stems and leaves developing into flowers, flower bud forming on flowers, flower forming on other flowers, and flowers with fewer petals than normal and without stamens and pistils. In *Dendrobium* Sonia 17 (Tee *et al.* 2008), *in vitro* flowers induced by BAP were smaller compared to *in vivo* flowers, with some flowers showing malformations including incomplete floral structures and variation in shape, color, and arrangement. In the present study, normal *in vitro* flowers exhibited complete floral structures with shapes, sizes, and colors similar to the *in vivo* flowers. However, some flowers that were induced prematurely on GA<sub>3</sub>-supplemented MS medium showed temporary abnormalities: smaller size, a lack of stamens and pistils, and white color without purple pigmentation. Such abnormalities suggest that different conditions might be required for the induction and development of normal flowers (Tee *et al.* 2008). In the present study, for example, increasing the length and age of the explants used as the micropropagated shoots produced a higher percentage of normal *in vitro* flowering than the shoot tips.

No seed formation was observed on micropropagated plants *in vitro*, consistent with observations of *Gentiana triflora* Pall. var. *axillariflora* (Zhang and Leung 2000). However, the micropropagated plants later formed seeds under *ex vitro* conditions that were similar in morphology and germinability to *in vivo* seeds. The absence of seeds from the *in vitro* plants could be caused by the lack of effective pollination or viable pollen due to limited nutrients supplied by the flower induction medium during pollen maturation (Jayaprakash and Sarla 2001).

Capitate and peltate glandular trichomes were found on the surface of leaves and flower petals. The function of these trichomes depends on their structure and position. Ultrastructural observations suggest that the large, spherical head structure of glandular trichomes located on the surface on both sides of leaves and at the tips of the abaxial surface of corollas probably plays a role in the secretion of essential oil or compounds to protect against predators and extreme environments, or to attract pollinators, as proposed by Werker (2000). Ioannidis *et al.* (2002) demonstrated a positive correlation between the number and volume of glandular trichomes per leaf area and total oil content, supporting the idea that glandular trichomes contributed to the essential oil production in *O. basilicum*.

The present study revealed that *in vitro* and *in vivo* leaves of the same age and position had similar numbers of capitate glands and peltate glands per leaf area on the adaxial and abaxial surfaces except for fewer capitate glands on the adaxial surface of *in vivo* leaves. However, peltate glands on *in vitro* leaves had more partially filled oil sacs, as indicated by their creased and wrinkled folds, compared to the *in vivo* leaves which had more fully filled oil sacs. Gang *et al.*



(2001) reported that the partially filled peltate glands of *O. basilicum*, which appeared wrinkled and creased, were the characteristic of immature glands, which normally became expanded on maturity as the oil sacs filled. Consequently, the creased and wrinkled folds of the peltate glands on the *in vitro* leaves might be indicative of their immaturity, so little essential oil was produced, leaving the oil sacs partially filled.

Essential oil at a level of 4.50% was extracted from *in vivo* leaves. This was higher than levels reported for 15 varieties of *O. basilicum* from Brazil and the USA (Vieira and Simon 2006), which ranged from 0.58% to 1.68%. While linalool was found as the dominant compound in three studied *O. basilicum* varieties in Poland (Wesołowska *et al.* 2012), the present study found methyl chavicol as the dominant compound. These conflicting results show that variability of essential oil content and composition in different populations of the same plant species can be attributed to varied agroclimatic conditions of the regions (Wesołowska *et al.* 2012), resulting in chemotypic variations within the species. A chemotype designation usually occurs because the plant is grown in a different area of the world or a distinct climate that alters the chemistry of the plant.

In the present study, *in vitro* plants initially had lower essential oil content (1.99%) than *in vivo* plants (4.50%), but the level increased to 2.38% after 18 wk of acclimatization under *ex vitro* conditions. In contrast, methyl chavicol content in *in vitro* plants (93.71%) initially was higher than in *in vivo* plants (66.29%), and the amount decreased to 60.07% after 18 wk of *ex vitro* acclimatization. Similarly, Verma *et al.* (2012) obtained the highest yield of essential oil at full bloom stage. The lower essential oil content of the *in vitro* plants compared to the *ex vitro* and *in vivo* plants could be a result of the younger developmental stage of the *in vitro* plants, which lacked fully developed glandular trichomes. Peltate glandular trichomes of the *in vitro* leaves in the present study seemed to be partially filled, resulted in reduced essential oil content.

The higher amount of methyl chavicol in the essential oil of the *in vitro* plants versus that of the *ex vitro* and *in vivo* plants indicated that methyl chavicol may be actively synthesized at the younger stage of development of the regenerated plants. Deschamps and Simon (2010) revealed that *O. basilicum* essential oil content and composition depended on plant developmental stage, whereby the accumulation of methyl chavicol decreased as leaves matured a result of the decreasing chavicol *O*-methyltransferase (CVOMT) transcript expression levels with leaf age. It was also noted that *ex vitro* plants showed an intermediate level of essential oil content between the *in vitro* and *in vivo* plants and a similar methyl chavicol content as the *in vivo* plants. This indicated that *ex vitro* plants were positioned at an intermediate stage of plant development, ready for the transition from the younger *in vitro* stage towards maturity, and had nearly the same essential oil content and methyl chavicol content as the more mature *in vivo* plants.

In conclusion, efficient protocols for micropropagation and *in vitro* flowering of *O. basilicum* from shoot tip explants were developed. The results suggested that BAP at a concentration of 1.0 mg L<sup>-1</sup> was important for inducing shoot proliferation, while GA<sub>3</sub> at concentration of 1.0 mg L<sup>-1</sup> was important for inducing *in vitro* flowering. Ultrastructural study showed that *in vitro* leaves and *in vitro* flowers could produce essential oil because of peltate glandular trichomes on their surfaces. Although the micropropagated plants under *in vitro* conditions showed some differences, such as flowers without seed formation, fewer fully filled peltate glandular trichomes, lower essential oil content, and higher methyl chavicol content compared to the *in vivo* mother plants, after they were acclimatized to *ex vitro* conditions, the micropropagated plants eventually displayed similar leaf and flower morphology, seed germinability, and methyl chavicol content as the *in vivo* mother plants.

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# Appendix IV

Poster presented

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## The effects of various hormones on *in vitro* regeneration and *in vitro* flowering of *Ocimum basilicum* L.



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### Introduction

*Ocimum basilicum* L., commonly known as sweet basil, is a herb belongs to the family Lamiaceae (Labiatae). Besides its ornamental use, this plant is also valued for its aromatic essential oil content. The aromatic leaves, flowers and seeds are included in foods, beverages, perfumes, cosmetics and traditional herbal remedies to treat cough, mental fatigue, fever, urinary ailments, constipation, diarrhea and migrain (Duke, 2001). Compounds in *O. basilicum* essential oil also have antioxidant, antiproliferative and antiviral properties (Jayasinghe *et al.*, 2003; Manosroi *et al.*, 2006 and Chiang *et al.*, 2005). *In vitro* regeneration is suggested to be an effective mean for rapid and large-scale propagation of plant with high progeny uniformity. The flowers produced *in vitro* can provide a convenient model to study the whole mechanisms of the reproductive process.

### Objectives

1. To study the effects of different plant growth regulator combinations and concentrations on the shoot proliferation in *Ocimum basilicum* for efficient regeneration system using shoot tip explants.
2. To study the effects of different plant growth regulator combinations and concentrations on the *in vitro* flowering in *Ocimum basilicum* for ideal reproductive model system using shoot tip explants.

### Results & Discussion

Table 1: Effect of different concentrations of plant growth regulators (PGRs) on shoot proliferation and *in vitro* flowering from shoot tip of *O. basilicum*.

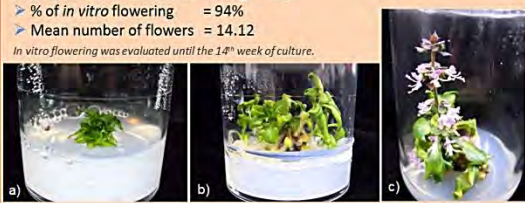
| Concentration of plant growth regulators (mg l <sup>-1</sup> ) |                     | % Shoot proliferation | Mean number of shoots per explant ± SE | Mean shoot length (cm) ± SE | % <i>in vitro</i> flowering ± SE |                       |
|--|---------------------|-----------------------|--|-----------------------------|----------------------------------|-----------------------|
| Cytokinin  | Auxin               |                       |  |                             |                                  |                       |
|  | 0                   | 23                    | 1.30 ± 0.11 <sub>a</sub>               | 1.83 ± 0.12 <sub>cd</sub>   | 20 ± 0.07 <sub>ab</sub>          |                       |
|  | 0.5 BAP             | 100                   | 3.60 ± 0.17 <sub>cd</sub>              | 1.26 ± 0.08 <sub>ab</sub>   | 3 ± 0.03 <sub>a</sub>            |                       |
|  | 1.0 BAP             | 100                   | 5.00 ± 0.28 <sub>e</sub>               | 1.42 ± 0.11 <sub>abc</sub>  | 0 ± 0.00 <sub>a</sub>            |                       |
|  | 1.5 BAP             | 100                   | 4.50 ± 0.29 <sub>de</sub>              | 1.41 ± 0.07 <sub>abc</sub>  | 0 ± 0.00 <sub>a</sub>            |                       |
|  | 2.0 BAP             | 100                   | 4.63 ± 0.19 <sub>e</sub>               | 1.19 ± 0.07 <sub>ab</sub>   | 0 ± 0.00 <sub>a</sub>            |                       |
|  | 0.5 BAP             | 0.5 NAA               | 77                                     | 2.33 ± 0.19 <sub>b</sub>    | 1.39 ± 0.13 <sub>bc</sub>        | 0 ± 0.00 <sub>a</sub> |
|  | 1.0 BAP             | 0.5 NAA               | 83                                     | 2.60 ± 0.18 <sub>b</sub>    | 1.17 ± 0.10 <sub>ab</sub>        | 0 ± 0.00 <sub>a</sub> |
|  | 1.5 BAP             | 0.5 NAA               | 93                                     | 3.20 ± 0.30 <sub>bcd</sub>  | 1.20 ± 0.09 <sub>ab</sub>        | 3 ± 0.03 <sub>a</sub> |
|  | 2.0 BAP             | 0.5 NAA               | 97                                     | 4.07 ± 0.27 <sub>de</sub>   | 1.14 ± 0.10 <sub>ab</sub>        | 0 ± 0.00 <sub>a</sub> |
| Gibberellin  | Auxin               |                       |  |                             |                                  |                       |
|  | 0                   | 60                    | 2.00 ± 0.19 <sub>ab</sub>              | 1.80 ± 0.15 <sub>cd</sub>   | 23 ± 0.08 <sub>ab</sub>          |                       |
|  | 1.0 GA <sub>3</sub> | 60                    | 2.07 ± 0.22 <sub>ab</sub>              | 2.16 ± 0.20 <sub>cd</sub>   | 40 ± 0.09 <sub>b</sub>           |                       |
|  | 1.5 GA <sub>3</sub> | 50                    | 2.17 ± 0.26 <sub>ab</sub>              | 2.73 ± 0.24 <sub>d</sub>    | 23 ± 0.08 <sub>ab</sub>          |                       |
|  | 2.0 GA <sub>3</sub> | 50                    | 2.17 ± 0.31 <sub>ab</sub>              | 2.16 ± 0.20 <sub>cd</sub>   | 20 ± 0.07 <sub>ab</sub>          |                       |

Shoot proliferation was evaluated after 8 weeks of culture with 30 replicates for each treatment. *In vitro* flowering was evaluated until the 20<sup>th</sup> week of culture with 30 replicates for each treatment. Values represent (mean or %) ± standard error of the mean followed by the same letter within columns are not significantly different (P=0.05) using Dunnett T3's multiple range test.

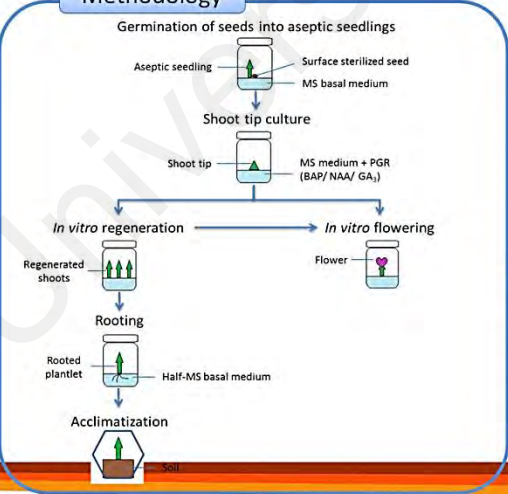
Using the *in vitro* regenerated shoot, *in vitro* flowering was induced on MS medium supplemented with 1.0 mg l<sup>-1</sup> GA<sub>3</sub>;

- % of *in vitro* flowering = 94%
- Mean number of flowers = 14.12

*In vitro* flowering was evaluated until the 14<sup>th</sup> week of culture.



### Methodology



### Conclusion

This work presents an efficient shoot proliferation and *in vitro* flowering methods for regeneration and reproductive model system in *Ocimum basilicum*;

- Using shoot tip explant, MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP was the best treatment for the optimum shoot proliferation in *Ocimum basilicum* (100% of shoot proliferation; mean number of shoots per explant = 5.00).
- MS medium supplemented with 1.0 mg l<sup>-1</sup> GA<sub>3</sub> was the best treatment for the optimum *in vitro* flowering in *Ocimum basilicum* (40% of *in vitro* flowering using shoot tip explant; 94% of *in vitro* flowering using *in vitro* regenerated shoot).


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## Appendix V

### Certificates of awards







**ITEX 13** 23<sup>rd</sup> INTERNATIONAL INVENTION, INNOVATION  
MALAYSIA & TECHNOLOGY EXHIBITION  
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## Certificate of Award

*This is to certify that*

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**IN VITRO FLOWERING FOR PRODUCTION OF FLORAL HANDICRAFT  
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**Kuala Lumpur, Malaysia**

**9<sup>th</sup> - 11<sup>th</sup> May 2013**



  
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