Establishment of an efficient protocol for the micropropagation of Holy Basil (*Ocimum sanctum* L.)

By

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ESTABLISHMENT OF AN EFFICIENT PROTOCOL FOR THE MICROPROPAGATION OF HOLY BASIL (OCIMUM SANCTUM L.)

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The focus of this thesis was to develop a micropropagation protocol and characterize the medicinal plant Ocimum sanctum L. An efficient system was established for in vitro multiplication of shoots (2.5 shoots/explant) using BA (1.1 μM) and GA₃ (0.3 μM). The addition of AIP, a phenolic pathway inhibitor, at 2 μM along with AC (0.6%) improved the formation of shoots (6.3 shoots/explant) and also alleviated the problem of liquification of culture medium. Microshoots, rooted in a medium containing 0.5 μM IBA with AC (0.6%), had a high survival rate (83%) when transplanted into the greenhouse. Assessment of antioxidant activity of 80 plants led to the selection of an elite plant named “Vrinda” for large-scale propagation. Morphological characterization of “Vrinda” showed a compact appearance of plants and delayed flowering compared to other populations. Phytochemical analysis of holy basil revealed the presence of neurotransmitters melatonin, serotonin and GABA.
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<tr>
<td>2, 4D</td>
<td>2,4-Dichlorophenoxyacetic acid</td>
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<td>AC</td>
<td>Activated Charcoal</td>
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<td>AIP</td>
<td>2-aminomide-2-phosphoric acid</td>
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<td>BA</td>
<td>6-Benzylaminopurine</td>
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<tr>
<td>DPPH</td>
<td>1-diphenyl-2-picrylhydrazyl</td>
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<td>FC</td>
<td>Folin–Ciocalteu Assay</td>
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<td>GA₃</td>
<td>Gibberellic acid</td>
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<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<tr>
<td>GAE</td>
<td>Gallic acid equivalent</td>
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<tr>
<td>IAA</td>
<td>Indole-3-acetic acid</td>
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<td>IBA</td>
<td>Indole-3-butyric acid</td>
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<td>iP</td>
<td>4-hydroxy-3-methyl-trans-2-butenylaminopurine</td>
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<td>KN</td>
<td>Kinetin</td>
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<tr>
<td>MEL</td>
<td>Melatonin</td>
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<td>MS</td>
<td>Murashige and Skoog basal salts</td>
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<td>NAA</td>
<td>Naphthalene acetic acid</td>
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<td>NHP</td>
<td>Natural Health Product</td>
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<td>PAL</td>
<td>Phenylalanine ammonia lyase</td>
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<tr>
<td>PCIB</td>
<td>p-Chlorophenoxyisobutyric Acid</td>
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<tr>
<td>SEM</td>
<td>Scanning Electron Microscope</td>
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<td>SER</td>
<td>Serotonin</td>
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<td>TE</td>
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<td>TDZ</td>
<td>Thidiazuron</td>
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<td>TIBA</td>
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1.0 Chapter 1: Introduction and Review of Holy Basil (Ocimum sanctum L.) as a Medicinal Plant

1.1 General Introduction

Self-reliance by consumers to take charge of their health using preventive, self-care strategies for non-critical conditions is a current and rapidly increasing global trend. Economic crisis and rising healthcare costs in North America have further promoted natural alternatives into becoming mainstream options for consumers in pursuit of better health. The global herbal market is estimated at about US$60 billion (Newmaster et al., 2013). Products of plants such as Stevia, Aloe, Milk thistle, Echinacea, and Ginseng alone are worth $60 million annually. A recent survey found that about 70% of Canadians use NHPs. Canadian NHPs industry generated revenues of C$2.9 billion including C$545 million in export to the health and wellness market abroad (Government of Canada, 2009).

Canada is home to an increasing ethnically diverse population and the demand for NHPs commonly used in traditional medicine systems of well-established cultures such as India and China is simply huge (Government of Canada, 2013a). With the greatest concentration of immigrants in the Greater Toronto Area and surrounding regions (Government of Canada, 2013b), the opportunities for locally grown traditional crops that are currently being imported are expected to rise significantly in the near future. However, authentic NHPs which will comply with new safety and efficacy regulations from Health Canada are not commonly available. In addition, the pharmaceutical and dietary supplement companies as well as the academic community
are intensely searching for novel NHPs to maintain or improve physical and mental health to alleviate the economic burden associated with illness. Ayurveda (the knowledge of life) is one of the oldest medical systems in the world, which prescribes medicinal plants to restore equilibrium within the body and the mind to rejuvenate health and to prevent the occurrence of complex diseases (Shekelle et al., 2005). A number of medicinal plants used in the traditional Ayurvedic medicines have significant potential to delay the occurrence of several chronic diseases and even cure them. Plants such as *Ocimum sanctum* L. are known as one complete pharmacy for preventing disease, promoting health, and prolonging life and provide an excellent opportunity to develop home grown NHPs for ethnic as well as local communities.

The objective of this study was to develop a model to introduce Ayurvedic plants and products in Canada as well as to develop a framework to commercialize new NHPs using *in vitro* and controlled environment production technologies. Holy basil was selected as the species of choice as this unique medicinal herb will be a novelty socially and economically and will be well accepted due to its proven multiple health benefits (Das and Vasudevan, 2006; Government of Canada, 2004a). Holy basil (*Ocimum sanctum* L.), also known as Tulsi or sacred basil, is renowned as one of the holiest and most cherished plants of India and other countries such as Nepal, Sri Lanka, and West Indies for its religious and spiritual sanctity (Engels and Brinckmann, 2013). Holy basil is considered a premier adaptogen capable of sustaining a balance of physiological and psychological functions of the body and mind through enhanced endurance against the physical, emotional, chemical and environmental stresses (Mohan et al., 2011). Leaves
of holy basil are consumed on a daily basis as tea, powders, and food additives in India for its antibiotic, antiviral, antifungal, antidiabetic, anticarcinogenic, immunomodulatory, hepatoprotective and adaptogenic properties (Prakash and Gupta, 2005; Mondal et al., 2009). Medicinal properties and various aspects of controlled environment production and characterization of holy basil plants are discussed below.

1.1.1 Holy Basil: The Elixir of Life

Holy basil is one of the most sacred herbs of India, and is an integral part of ancient Hindu traditions. According to Hindu mythology, Holy basil originated as one of the 14 “Ratnas (gems or treasures)” from the ocean as the ultimate sacred plant to enhance health and remove diseases. Holy basil is believed to be a manifestation of the Goddess Lakshmi, the wife of God Vishnu, and is worshiped for health, wealth, and happy married life (Raina et al., 2013). Necklaces made from holy basil stems or roots are auspicious to devotees who wear them around their neck or wrist to seek God’s blessings. The plant is considered a blessing from God, a religious symbol, and a magic herb as described in many ancient medicinal texts such as Ayurveda, Siddha and Unani (Engels and Brinckmann, 2013). Thus, holy basil has been a permanent fixture in Hindu homes, temples, and sacred shrines and its Sanskrit name “Tulsi” (a word for “the incomparable or matchless one”) truly defines the significance of this legendary Ayurvedic plant used since time immemorial.

Traditional uses of holy basil as medicine usually include, but are not limited to the treatment of: abdominal issues, oral infections, cough, colds, tumours and cancer, digestive tract problems, respiratory inflammation, arthritis, asthma, ulcers, wounds, hypoglycaemia, bronchitis, urinary issues, cardiovascular problems and many others
Das and Vasudevan, 2006; Mohan et al., 2011; Engels and Brinckmann, 2013; Kumar et al., 2013; Mondal et al., 2009; Singh and Verma, 2010). Although the whole plant is medicinally important, the leaves are generally the most common form of treatment, and are either used raw or steeped in hot water. The medicinal properties of holy basil leaves include: antimicrobial, anticancer, antistress, adaptogenic, stimulant, expectorant, nervine, antipyretic and antiperiodic (Engels and Brinckmann, 2013; Government of Canada, 2004a). These beneficial properties have led to holy basil being named “Queen of Herbs”, “Incomparable One” and “The Mother Medicine of Nature” and one of the most valued medicinal and religious herbs in India (Singh and Verma, 2010). The knowledge of this traditional medicinal herb is expanding to other cultures due to its unique therapeutic properties.

1.1.2 Botany

Holy basil (Ocimum sanctum L.) is included in the family Lamiaceae, along with several other medicinal herbs such as salvia and mint (Gershenzon et al., 1992; Baran et al., 2010) and over 65 species of basil that are used in the food, pharmacology and perfume industry (Trevisan et al., 2006; Makri and Kintzios, 2008). The species is closely related to sweet basil (Ocimum basilicum), a commonly used herb in Europe and North America. Holy basil has also been described as Ocimum tenuiflorum (basil with small flowers) or Ocimum gratissimum (very grateful basil). Of these, the species name O. sanctum is favoured due to the wide range of its uses in religious and cultural traditions (Engels and Brinckmann, 2013).
Three distinct forms of holy basil are commonly distributed throughout the Indian subcontinent: 1) Sri or Rama Tulsi with green leaves; 2) Krishna Tulsi with dark green to purple leaves; and 3) Vana Tulsi with green leaves but the plant grows in the wild (Engels and Brinckmann, 2013). Of these, the Rama and Krishna holy basil are most commonly grown in homes and commercial production for use in Ayurvedic preparations. The plant is found mainly in subtropical and tropical areas of Asia including India, China, Malaysia, Sri Lanka, and Thailand in addition to Australia and Africa, at altitudes of up to 1800m in India to sandy dry conditions in China (Engels and Brinckmann, 2013; Kumar et al., 2013).

1.2 Morphology

Holy basil is an upright plant with many branches and can grow from 20 to 150 cm in length supported by a square stem that is hairy and lignified at the base (Mondal et al., 2011; Mohan et al., 2011; Gupta et al., 2002; Engels and Brinckmann, 2013; Das and Vasudevan, 2006; Jürges et al., 2009). The leaves of the plant are highly aromatic, similar to clove, and are arranged opposite, alternate, and found to be elliptic or ovate in shape, with hairs on both adaxial and abaxial sides and margins that are toothed, serrated or entire (Gupta et al., 2002; Jürges et al., 2009; Kumar et al., 2013). The inflorescences of the plant are long cylindrical racemes purple in colour. The flowers are in compact whorls with bell shaped petals, 60-100mm in length and produce small yellow-brown fruits (Kumar et al., 2013; Gupta et al., 2002; Jürges et al., 2009).
1.3 Production of Holy Basil

Holy basil is an important part of the agricultural system in India and is among one of the 178 highly cultivated plant species and one of only 36 species with stabilised local agricultural systems in India (Ved and Goraya, 2008). The consumption of holy basil within India is approximately 3500MT annually, with trade requirements between 2000 – 5000 MT, sourced through traditional cultivation practices (Engels and Brinckmann, 2013).

Basil plants have small seeds that require friable, well tilled soil and which are planted in late spring (April – May). Germination of seeds, planted in rows 10-15cm apart and with plants 2cm apart, occurs after 4 - 14 days (Darrah, 1974). It is common to see variation in the production and appearance of the plants based on the environmental alterations. Using a fertilizer with a 1:1:1 ratio of P, K, N is desirable, and the species is not water tolerant so a consistent, slow application of water is ideal (Darrah, 1974). The plant grows well under long days and high temperatures, preferably in full sun, but can tolerate partial shade (Kew, 2004). Removal of the inflorescence enhances vegetative growth and results in a flush of new leaves throughout the growing season. In tropical climate, the first holy basil crop can be harvested in about three months and under optimized growth conditions, as many as three to four harvests are easily possible. The method of harvest affects overall yield of essential oil content as well as individual components of the oil. For example, secondary branches were found to have decreased biomass but increased essential oil content with high levels of eugenol, and low levels of methyl-eugenol – two main chemical components of holy
basil. The biomass harvested from above 30cm from ground had decreased essential oil yield (Kothari et al., 2004).

1.4 Medicinal Properties of Holy Basil

Medicinal effect on holy basil is believed to be due to the complexity of the constitutes in the plant, leading to many positive influences in the human body. Innumerable medicinal benefits of holy basil have been recorded in many different regions and local languages of India. Recently, scientific evidence of therapeutic effect of holy basil has started to emerge in mainstream medical journals mostly from studies using in vitro bioassays and small clinical trials. A few recent examples of such studies on holy basil include: antimetastatic activity against Lewis Lung carcinoma cells (Magesh et al., 2009), cardioprotective inhibition of lipid peroxidation in rats induced with myocardial infarction, and high cholesterol or high cadmium diets (Sharma et al., 2001; Suanarunsawat et al., 2010; Ramesh and Satakopan, 2010), immunostimulation through elevation in levels of TNF-α, IFN-γ and IL-2 cytokines in rats infected with Salmonella typhimurium (Goel et al., 2010), neuroprotection and normalization of brain function through modulation of neurotransmitters (Yanpallewar et al., 2004; Ahmad et al., 2012; Muthuraman et al., 2008; Ravindran et al., 2005) and prevention of radiation mediated cell death in mice (Subramanian et al., 2005; Ganasoundari et al., 1997). Other examples of the medicinal properties of holy basil are antimicrobial benefits as a mouth rinse (Agarwal and Nagesh, 2011), inhibition of bacterial gonorrhoea (Shokeen et al., 2008), reduced acne (Viyoch et al., 2006), anthelmintic activity (Asha et al., 2001), wound healing (Shetty et al., 2008; Goel et al., 2010) and reduced levels of plasma glucose (Agrawal et al., 1996; Chattopadhyay, 1993; Vats et al., 2004, 2002),
triglyceride and cholesterol (Rai et al., 1997). Holy basil can also reduce diabetic symptoms and blood pressure (Kochhar et al., 2009) and stimulate insulin production in the pancreas (Hannan et al., 2006) as well as, subdue skin, breast, and gastric cancer because of the antioxidative properties (Baliga et al., 2013).

In numerous rat studies, the inclusion of holy basil improved the stress response through normalizing changes and associated oxidative damage induced by stresses such as noise, restraint and an increased swimming time (Archana and Namasivayam, 2000; Gupta et al., 2007; Maity et al., 2000; Samson et al., 2007; Tabassum et al., 2009; Bathala et al., 2012; Sood et al., 2006).

Holy basil has been described to improve symptoms of disorders such as Alzheimer’s or dementia, anxiety (Bhattacharyya et al., 2008), depression and cerebral reperfusion (Yanpallewar et al., 2004) indicating its anti-stress influence on the central nervous system (Joshi et al., 2011; Pemminati et al., 2010; Yanpallewar et al., 2004; Khanna and Bhatia, 2003; Bhattacharyya et al., 2008).

Although the most widespread use of holy basil remains to be medicinal, recent studies have shown that this plant is effective against a variety of biological pollutants present in water (Sundaramurthi and Dhandapani, 2012; Parag et al., 2010). Holy basil essential oils have antifungal and anti aflatoxigenic effects which can be used for storage and improvement of shelf life of food products such as ‘Tofu’ (Kumar et al., 2010; Anbarasu and Vijayalakshmi, 2007). There is evidence that holy basil extracts can improve the shelf life of bananas and may also be a viable alternative to chemical fungicides for the management of crown rot disease (Sangeetha et al., 2010). Strong
antimicrobial effects of the essential oil constituents against pathogenic fungi and both gram-positive and gram-negative bacteria have also been demonstrated (Pandey and Madhuri, 2010; Amber et al., 2010).

1.5 Chemistry:

A wide range of technologies have been used for the quantification of the chemical composition as well as antioxidant potential of holy basil including gas chromatography (GC), high performance thin layer chromatography (HPTLC), GC-mass spectrometry (GC-MS), 1D and 2D NMR spectroscopic analysis, solid phase microextraction (SPME), flame ionization detection (FID), and olfactory evaluation (Amber et al., 2010; Pandey and Madhuri, 2010; Gupta et al., 2007; Jirovetz et al., 2003). Antioxidant potentials have been measured separately by assays such as DPPH (1-diphenyl-2-picrylhydrazyl), and TBARS (thiobarbituric acid reactive substances) or have been combined with HPLC (high performance liquid chromatography) (Trevisan et al., 2006). Techniques such as x-ray fluorescence analysis and Fourier transform infrared spectroscopy coupled with principal component analysis have been used to evaluate the uniformity and identity of herbal plants for quality control purposes (Singh and Verma, 2010; Morabad and Kerur, 2010).

The chemical composition of holy basil analyzed by multiple techniques described above revealed a highly complex mixture of many nutrients, essential oils and other biologically active compounds. The plant contains a diverse range of compounds including alkaloids, carbohydrates, fats, flavonoids, glycosides, phenols, proteins, saponins, tannins and terpines (Engels and Brinckmann, 2013; Kumar et al., 2013). The most common compounds present in high quantities include, eugenol, methyl-eugenol,
ursolic acid, rosmarinic acid, linalool, caryophyllene, carvacrol, alpha-terpineol and alpha-bisabolene (Kumar et al., 2013; Singh and Verma, 2010; Singh et al., 2012; Kothari et al., 2004; Prakash and Gupta, 2005; Sundaramurthi and Dhandapani, 2012; Mondal et al., 2009; Raina et al., 2013; Iracema et al., 1999). Generally, the main components of the leaf tissue are eugenol and methyl-eugenol but their levels vary in different plants (Prakash and Gupta, 2005; Trevisan et al., 2006; Viyoch et al., 2006; Asha et al., 2001). These constituents along with the numerous other compounds found in holy basil at lower levels, give rise to the variety in medicinal properties.

The medicinal properties of eugenol include a positive influence on the immune, reproductive, central nervous, cardiovascular, gastric and urinary tract systems (Wangcharoen and Morasuk, 2007) and other activities such as being anthelmintic (Asha et al., 2001), antigonorrhoea (Shokeen et al., 2008), anticancer (Pandey and Madhuri, 2010; Baliga et al., 2013), and reducing cholesterol, triglyceride and blood sugar (Prakash and Gupta, 2005; Holm, 1999). Methyl-eugenol is another phenolic compound found in holy basil, but at lower quantities (20%) (Baliga et al., 2013). Usually more commonly used as a flavouring agent or in the perfume industry due to its’ strong woody, spicy flavour (Raina et al., 2013; Kothari et al., 2004), the compound also has some similar properties as eugenol. These include: antibacterial, insecticidal, anthelminthtic, stimulant and anaesthetic (Raina et al., 2013; Viyoch et al., 2006).

Another major component with medicinal qualities is ursolic acid, an isomer of oleanolic acid as a saponin (Liu, 1995). Found in a variety of medicinal plants, it is known to have many useful properties such as, antiinflammation, hepatoprotection, antihyperlipidemic, antimutagen, antioxidant, antidepressant, neuroprotection, anxiolytic, antimicrobial,
decrease lipid peroxidation and protective against oxidative damage in cells (Sundaram et al., 2012; Liu, 1995; Ovesná et al., 2006). Rosmarinic acid and caryophyllene are also important constituents of holy basil extract and have an assortment of medicinal properties including antiviral, antimicrobial, antiinflammatory and antioxidant effects (Pandey and Madhuri, 2010; Chanda et al., 2012; Petersen and Simmonds, 2003; Makri and Kintzios, 2008; Raina et al., 2013; Viyoch et al., 2006). Holy basil leaves are rich in minerals such as calcium, zinc, phosphorus, iron and also contain vitamin A and C (Sundaramurthi and Dhandapani, 2012).

1.6 Antioxidants in Plants

Antioxidants scavenge free radicals within the body and externally. Free radicals are unstable and react with cell membranes, proteins, DNA and lipids causing structural and functional damage (Halliwell, 2009). This is undesirable as it can lead to premature aging of the cells, causing degenerative diseases, cancer and cardiovascular problems and other health issues (Bondet et al., 1997). Antioxidants provide protection by offering a free proton and are oxidized themselves, thereby stopping the oxidation chain reaction (Juntachote and Berghofer, 2005).

To assess plants for their antioxidant abilities, the use of various analytical methods can be used; one such popular method is the DPPH (1-diphenyl-2-picrylhydrazyl) free radical scavenging assay. DPPH is a stable free radical, after the addition of the sample to the radical, a colour change occurs (from purple to yellow) and the DPPH accepts hydrogen atoms from the antioxidants in the sample (López-Alarcón and Denicola, 2013). This method has been used in evaluation of numerous medicinal
and food plants, including holy basil, to determine their antioxidant activity (Ramesh and Satakopan, 2010; Juntachote and Berghofer, 2005; Trevisan et al., 2006; Hakkim et al., 2007; Wong et al., 2006; Wangcharoend Morasuk, 2007; Lee et al., 2003).

1.7 Neurotransmitters as Antioxidants: Melatonin, Serotonin and GABA

Neurotransmitters are recently being discovered in plants with high antioxidant values to assist with plant abiotic and biotic stress. Non-vertebrates, plants, bacteria and fungi have recently been discovered to contain compounds originally believed to be only found in vertebrates (Chen et al., 2003). These compounds are neurotransmitters in humans and include serotonin (5-hydroxtryptamine), melatonin (N - acetyl – 5 – methoxytryptamine) and GABA (υ - aminobutyric acid) (Kinnersley and Turano, 2000; Roberts, 2007; Mohammad-Zadeh et al., 2008; Chen et al., 2003). Neurotransmitters have been extensively studied in humans, and are believed to have beneficial properties in the body by acting as an antioxidant, regulate circadian rhythms by improving sleep and jet lag, inhibiting migraines, improve depression and mood disorders, relieve symptoms of irritable bowel syndrome, and improve epilepsy, hypertension and gastrointestinal problems, in addition to working in the central nervous system as signalling cascades (Mohammad-Zadeh et al., 2008; Stafford, 2005; Cole et al., 2007; Reiter and Tan, 2001). In plants, these compounds have been discovered to play a similar role in signalling, acting as an antioxidant protectant, inhibiting stress on the plant and maintaining stability of internal levels such as pH, metabolism and osmoregulation (Bouché and Fromm, 2004; Reiter and Tan, 2001; Badria, 2002). Neurotransmitters may function like an adaptogen in the plant, similar to how holy basil acts as a adaptogen in the human body.
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Plants have recently been discovered with levels of these compounds at significant quantities (Badria, 2002; Murch et al., 1997; Murch and Saxena, 2006; Murch et al., 2004; Chen et al., 2003). Although not previously found in holy basil, the properties and the vast range of chemical components found in this medicinal species may indicate significant levels of neurotransmitters in the plant. Currently, melatonin, serotonin and GABA are approved in the Canadian system of natural health products (Government of Canada 2004a,b,c) and, if present in holy basil, would be a boon for the industry.

1.8 Natural Health Products: Canadian context

In Canada, holy basil would be classified as a Natural Health Product (NHP) as it is a traditional medicine. Canada’s natural health industry is growing, with 70% of Canadians currently using a natural health product, including herbals, homeopathic medicine or vitamins and minerals (Government of Canada, 2004e). Worldwide, numerous plants (13 000) are described as being traditional medicines that are used by 90% of the world’s population (Agri-Food Canada, 2012). This industry is also constantly growing in Canada with a 15% rise in revenue for Canadian companies over two years estimated at approximately $2.9 billion dollars (Government of Canada, 2009). However, increasing interest in natural remedies has also brought about the great challenge of maintaining a balance between the demand of expanding markets for plant-based medicines and the quality of the products. Recent studies of Ayurvedic products in the USA raised safety concerns related to toxicity of formulations, drug interactions and lack of scientific evidence. A study funded by the National Center for Complementary and Alternative Medicine (NCCAM) (Anonymous, 2009) found that of
the 70 Ayurvedic remedies purchased over-the-counter (all manufactured in South Asia), 14 contained lead, mercury, and/or arsenic at levels that could be harmful to human health. In 2004, the Center for Disease Control and Prevention reported 12 cases of lead poisoning linked to the use of Ayurvedic medications (Anonymous, 2009). Health Canada also has issue warning against the use of certain Ayurvedic medicinal products because they contain high levels of heavy metals such as lead, mercury and/or arsenic. Other concerns of Ayurvedic medicines include adulteration with poor substitute plants and wide variation in bioactive compounds (Newmaster et al., 2013).

In Canada, several regulations are now in place to ensure the safety of the products for consumers. These include requirements of the evidence of quality, safety, and efficacy through good manufacturing practices (GMP), clinical trials and proper labelling and packaging (Government of Canada, Health Canada, 2013). Canada’s strong role in upholding these standards, and the advancements in technology to identify consistency of active ingredient levels ensures consumer safety and efficacy of the product (Agri-Food Canada, 2012). However, issues of contamination and authenticity of NHPs are still prevalent despite these strict guidelines. Recent research in Canada found that some of the NHPs were of poor quality containing unlabelled plants, fillers, and contaminations and/or substitution with other plants which may be toxic (Newmaster et al., 2013). Thus, producing the NHPs locally in Canada rather than outsourcing from other countries with different regulations is highly desirable for maintaining the integrity of medicinal plant products. Additionally, the production of multipurpose plants such holy basil, which is used in religious as well as medicinal
purposes and can also be introduced as an ornamental, may enhance competitiveness of Canadian plant industries.

1.9 Clonal Propagation using *In Vitro* Methods

The nutritional and pharmacological properties of medicinal plants are a result of synergistic interactions of many different phytochemicals. However, the amount of phytochemicals varies with cultivars and is also significantly affected by growing, harvesting, processing and storage conditions and the flavour and taste of the extracts are strongly influenced by weather. This inherent biochemical complexity makes it necessary to standardize the overall growth and physiology of the plants to maintain uniform chemical profiles of the tissues used in medicinal preparations to achieve optimum effect.

To clonally propagate plants with high medicinal value, the use of controlled environment systems is ideal due to the highly regulated growth conditions of light, water, temperature, soil and other factors which also maintain the good manufacturing procedures required by Health Canada. Controlled environment systems include both the use of greenhouses and *in vitro* techniques to grow plants. *In vitro* propagation of plants is the use of a sterile, controlled environment in which plants are provided all the necessary nutrients through a pathogen free medium (George et al., 2008). *In vitro* propagation has been previously used in other medicinal plants such as salvia, St. John's Wort and ginseng (Murch and Saxena, 2006; Makunga and Staden, 2007; Uchendu et al., 2011). As such, tissue culture provides a unique tool as it can be more efficient at maintaining numerous plant species or germplasm lines at a young growth
stage which can be later transferred into outdoor or greenhouse environments (George et al., 2008). Manipulation of media components and external inputs can increase production of key medicinal constitutes or tissue types (Rout et al., 2000).

1.9.1 Establishment of Cultures

Establishing a protocol to regenerate, multiply and clone plants in vitro requires establishment of a sterile cultures of meristematic tissues such as shoot tips and nodal segments which can be obtained from seeds germinated in vitro or from field or greenhouse grown plants. The process of culture establishment is accomplished by surface sterilization to remove pathogens using alcohol, calcium hypochlorite or mercuric chloride and many others (George et al., 2008). Surface sterilization is followed by washing and transfer to growth medium containing essential nutrients such as water, sugar, micro and macro nutrients, and plant growth regulators to optimize growth in vitro (George et al., 2008). Many media compositions have been previously established based on basic plant requirements, but the optimized ratios and concentrations of those components are usually at least slightly variable based on tissue type, species and method of propagation (Rout et al., 2000).

Establishment of cultures in vitro is not particularly challenging for holy basil and both seed and explants have been cultured with high success rates with no significant contamination. Seeds were successfully germinated on moistened filter paper on Petri plates (Mathew, 2011; Tyub and Kamili, 2009) or on solidified media containing MS (Murashige and Skoog, 1962) salts, with a combination of BA (6-benzylaminopurine) (4.43 µM) and IAA (indole-3-acetic acid) (0.57 µM) (Gogoi and Kumaria, 2011). Leaf,
axillary buds or inflorescence from plants grown in field or in greenhouse are more commonly used explants to establish sterile cultures.

Pre-sterilization washes were done in some of the studies and were variable in concentration and time. For example, mature shoot and leaf tips were washed with water and salvon (1%) for 20 minutes prior to sterilization (Banu and Bari, 2007) and axillary buds with a 5 minute rinse of laboline and sodium hypochlorite, followed by a water rinse before sterilizing (Mandal et al., 2000). Pattanaik and Chand (1996) also used axillary buds and only did a prewash with water prior to surface sterilization with a combination of sodium hypochlorite, sodium chloride, sodium hydroxide and sodium carbonate for 10 minutes before rinsing with sterile water.

A prewash with water and detergent was done on inflorescences before surface sterilization and was found to require a medium containing MS salts and 2, 4D (2,4-dichlorophenoxyacetic acid) at different concentrations for explant establishment in culture (Singh and Sehgal, 1999). Leaf and inflorescences explants were shown to require MS salt media with different plant growth regulators to survive establishment after prewash with water before sterilization (Shilpa et al., 2009; Hakkim et al., 2007).

By contrast, Xiong Lim et al., (2009) employed a 30 minute pre-rinse followed by a 25% Clorox bleach solution for 10 minutes for culturing leaves on a basal medium. None of these studies provided data for the contamination rates or survivability of explants after sterilization, indicating that contamination and death of tissue are minor issues in holy basil culture possibly due to its natural antimicrobial properties inhibiting infection. After establishment of sterile cultures, the manipulation of media to increase multiplication for clonal propagation can be investigated.
1.9.2 Shoot Proliferation and Multiplication

For improving cloning methods, deriving multiple plantlets from an individual explant is determined as the multiplication rate. This process usually requires the addition of various plant growth regulators to stimulate shoot development and multiplication of the plants. Plant multiplication can be through direct or indirect shoot proliferation, or stimulation of nodal explants. Nodal explants with axillary buds elongate and form shoots in vitro, which can be subcultured for further cloning of plants (George et al., 2008). Adventitious shoots can also be derived directly from explants or indirectly through callus cultures. Indirect reproduction is generally unfavourable for medicinal plant propagation, as the callus formation can lead to genetic instability, and somaclonal variation.

The shoot cultures are maintained in media generally supplemented with naturally occurring cytokinins such as kinetin (KN), zeatin, and iP (4-hydroxy-3-methyl-trans-2-butenylaminopurine) or synthetic cytokinins, e.g., BA and thidiazuron (TDZ) (Jones et al., 2007). Cytokinins generally counter the apical dominance from auxins, and encourage cell division, differentiation, increase adventitious shoot and leaf development, and decrease rooting (Taiz and Zeiger, 2010). For tissue culture, the concentration or source of the cytokinin required varies with plant species, age and growth stage. This is due to the variation in endogenous levels of hormones affecting the requirements to encourage multiplication (George et al., 2008). Cytokinins may also function in combinations with other growth regulators such as gibberellic acids or auxins depending on the individual plant species or genotypes.
The use of nodal, direct and indirect methods has been explored using various explant materials in holy basil for shoot initiation. In this review both methods will be discussed, but for this thesis, the goal is to establish plants through nodal cultures, as there is less risk of genetic variability through somaclonal variation.

1.9.2.1 Indirect Shoot Regeneration

Various explants of holy basil such as inflorescence, nodal, leaf, stem, shoot tip, axillary buds and cotyledons have been used to initiate callus cultures using BA or TDZ (Singh and Sehgal, 1999) or 2,4-D (1.0 mg/L) in combination with kinetin (Hakkim et al., 2007). Nodal segments were induced to form callus and shoots with the combination of auxin and cytokinins, NAA (naphthalene acetic acid) (5 mg/L) and BA (0.5 mg/L) or 2,4D at 0.2 mg/L (Shahzad and Siddiqui, 2000) similar to multiplication from shoot tips with NAA 0.1 mg/L and BA at 0.2 mg/L (Banu and Bari, 2007) and axillary buds with NAA and kinetin (Gogoi and Kumaria, 2011). Cotyledons have also been used as the explant source for callus formation on medium with 2, 4D and BA (Mathew, 2011). Leaf explants are the most common source for callus initiation in holy basil using auxin-cytokinin combinations (Shilpa et al., 2009; Hakkim et al., 2007; Xiong Lim et al., 2009; Banu and Bari, 2007).

Callus formation in holy basil is quite varied in the response based on explant and plant growth regulators tested. However, the source material and time of harvest may play an important role in determining optimal levels of growth regulators for indirect callus formation. In some of these studies, the callus was further used to induce adventitious shoot regeneration. Callus from axillary buds required high levels of auxin
(NAA at 12.42 µM) with low levels of cytokinin (2.32 µM of KN) to increase shoot buds, length and leaf number (Gogoi and Kumaria, 2011). However, callus transferred onto cytokinin medium with 0.2 mg/L BA had 80% shoot regeneration (Banu and Bari, 2007). Similarly, nodal explant callus formed multiple shoots with media containing BA (5 mg/L) with low levels of auxin (NAA 0.2 mg/L) in addition to glutamic acid at 50 mg/L (Shahzad and Siddiqui, 2000). As well, cotyledon callus was used to initiate somatic embryogenesis with kinetin and IAA (Mathew, 2011).

1.9.2.2 Direct Shoot development from Explants

Direct shoot regeneration without an intervening callus from an explant is more desirable for propagating elite lines of a highly medicinal individual plant as the genetic stability is maintained. As with indirect regeneration, different explant sources have been used to initiate shoot response in vitro, including, peduncle, axillary buds, shoot tips, and nodal explants of holy basil. A higher ratio of cytokinin to auxin, or no additional auxin, in the medium is optimal for the majority of the explant material.

For example direct shoot formation from inflorescence was achieved with BA (4.4 µM) in combination with IAA at low concentrations (0.05 mg/L) (Singh and Sehgal, 1999) whereas axillary buds showed optimum response with BA at 4.4 µM (Mandal et al., 2000). Further enhancement in BA (4.4 µM) induced axillary bud proliferation was achieved with the addition of gibberellic acid at 0.5 mg/L (Pattnaik and Chand, 1996). In shoot tips and nodal explants, a combination of two cytokinins BA (4.4 µM) and KN (9.4 µM) was required to optimize shoot production (Girija et al., 2006).
1.9.3 Rooting and Hardening of Microshoots

Once established and multiplied, *in vitro* developed plants need to be hardened, or acclimatized before transfer to greenhouse conditions. Acclimatization is necessary to gradually reduce the influence of optimized environments provided for *in vitro* growth including high humidity, all the essential nutrients, no invasive species or pathogens, little to no competition, optimized light and ideal temperatures (George et al., 2008). The establishment of roots on *in vitro* shoots is the first step in ensuring acclimation as it allows plants to seek nutrients and water in soil while maintaining structural stability (George et al., 2008).

Auxins are known to induce lateral and adventitious root development, tropism, bud growth and the apical dominance, elongation, embryonic development, vascular tissue growth and phyllotaxis (Woodward and Bartel, 2005). Naturally occurring auxins including IAA and indole-3-butyric acid (IBA) as well as synthetic analogue such as NAA are commonly used to encourage root production *in vitro*. Concentrations and ideal auxin sources, whether natural or synthetic, are dependent on the plant species, and endogenous hormone levels. Optimum concentrations of auxin and cytokinins need to be determined to promote adventitious root growth, as the ratios of auxin to cytokinin can significantly influence root development (Rout et al., 2000). For holy basil, both direct and indirect formation of roots has been observed with and without the addition of plant growth regulators (Shilpa et al., 2009; Pattnaik and Chand, 1996; Girija et al., 2006; Shahzad and Siddiqui, 2000; Banu and Bari, 2007).
1.9.3.1 Indirect Root Formation

Indirect root formation in holy basil rhizogenic callus was obtained from leaf explants cultured on medium containing 2 mg/L of NAA with 0.2 mg/L KN (Shilpa et al., 2009). Transfer of this callus onto solid media containing 1.5 mg/L 2, 4D with 0.5 mg/L NAA, or the removal of cytokinins, resulted in optimized root development after three weeks. Afterwards, transfer of callus to liquid medium, containing high levels of NAA (1.3 mg/L) with the addition of BA (1.3 mg/L) increased the root production. This study demonstrated that both callus and roots can be generated using liquid culture.

1.9.3.2 Direct Root Formation

Direct root formation on micropropagated shoots of holy basil also requires a higher auxin to cytokinin ratio and is accomplished by the addition of NAA. Shoots derived from axillary buds were found to root optimally in media containing 1.0 mg/L NAA, with 98% root formation after 10-12 days (Pattnaik and Chand, 1996; Girija et al., 2006). A similar concentration of NAA (1.5 mg/L) was used in shoot explants derived from callus tissue (Shahzad and Siddiqui, 2000). It was also observed that 90% root formation was achieved with considerably lower concentrations (0.1 and 0.2 mg/L) of NAA than those reported previously (Banu and Bari, 2007). The use of auxins in combinations with cytokinins also produced significant root production in shoots. Concentrations of NAA at 26.85 µM and KN at 2.32 µM had improved root formation over other auxin (IAA) and cytokinins (BA) (Gogoi and Kumaria, 2011). Interestingly, shoots from inflorescence explants were observed to root best in media containing no additional hormones (Singh and Sehgal, 1999). With both direct and indirect methods
for root formation in holy basil, high success rates were obtained. Overall, the use of high auxin to cytokinin ratios, specifically the use of the auxin NAA, improved root production which is a common observation in most plant species.

1.9.4 Acclimatization and Transfer to Greenhouse

*In vitro* raised plants often need acclimatization prior to transplant in greenhouse or field conditions as they are adjusted to *in vitro* conditions of high humidity and abundant resources. Thus, plants may have poorly developed stomata on leaves and may not be completely autotrophic due to sugar supply in the media (George et al., 2008). *In vitro* grown plants are generally removed from culture conditions, rinsed and transferred to soil in the greenhouses with high humidity through misting and low light intensity. After a slow acclimatization through reducing humidity and increasing the light intensity, plants become fully autotrophic and established to survive independently.

In holy basil, survival rates of *in vitro* grown plants are generally high and with a short acclimatization process. Transplant of longer shoots with roots into soil containing vermiculite and garden soil at a 1:1 ratio, showed 85% survival after 2 weeks (Singh and Sehgal, 1999). In another acclimatization method by Pattnaik and Chand (1996), plants were transferred into growth chambers with high humidity for 2 weeks prior to greenhouse transfer with survival rates of 75 - 80% in the growth chamber, 80-85% after transfer to greenhouse followed by a 100% survival in natural conditions. Another study found a variable survival rating in different soil compost mixtures for holy basil, ranging from 12 to 82% with highest success rate after four weeks in soil and cow dung and the lowest survival in all other forms of compost (Gogoi and Kumaria, 2011).
1.9.5 Additional factors affecting Micropropagation

Medicinal plants respond normally to commonly used micropropagation treatments but some variations are possible. Browning of cultures is common due to high levels of phenolics in medicinal plants. By using polyphenol inhibitors, absorbents or through frequent subculturing the occurrence of browning can be alleviated (Rout et al., 2000). *In vitro* culture environment conditions are also a factor in the propagation process. A pH of 5.5 - 6.0 in the media allows for optimal absorption of nutrients which may change in autoclaving or due to the uptake and release of compounds by the plant (George et al., 2008). Gelling agents such as gellan gum or agar to create a semisolid matrix for plants to remain upright in culture but can affect plant health (George et al., 2008). Environmental conditions including the quality of light (35-112µmolm⁻²s⁻¹) and day length (12-16h) with a temperature range between 22-25°C are considered reasonable for tissue culture (Rout et al., 2000).

A unique process for large-scale production of medicinal plants is the use of bioreactors, which provide a self-contained, sterile growth environment for large-scale propagation of shoots, roots and whole plants in a short time frame. Medicinal plants can be rapidly multiplied in various air-lift bubble-type bioreactors or temporary immersion bioreactors (Murch et al., 2008; Jones et al., 2009; Abbasi et al., 2009). These bioreactors use a liquid rather than solidified media method to promote biomass growth and the production of secondary metabolites (Paek et al., 2005). Different designs of bioreactors are currently available including temporary immersion systems that allow for easy replacement of media, decrease cost of labour and space, increase secondary metabolite production and multiplication of plants (Paek et al., 2005; Thatoi
Bioreactors have been used in medicinal plants such as ginseng, garlic, St. John’s Wort and sweet basil (Paek et al., 2005; Zobayed and Saxena, 2004; Kintzios et al., 2004), but mass production in holy basil plants with liquid media has not been explored except for root development (Shilpa et al., 2009).

1.10: Conclusion

Holy basil is a unique medicinal herb with an exceptionally broad range of health-promoting, disease-preventing and life-prolonging attributes which have been documented over centuries (Engels and Brinckmann, 2013). The Canadian market for NHPs is expected to rise with an increasing ethnic population. Well recognized plants such as holy basil offer tremendous growth potential for the Canadian NHP industries. However, there are many challenges in the production of plant based medicines which put the consumer at serious health risk. Natural plant medicines of high commercial value are often prepared from plant materials harvested without sufficient knowledge of plant growth, physiology, propagation, or true origin which results in poor quality of the products. It is noteworthy that new regulations developed to ensure the safety and efficacy of NHPs released in Canada will now require scientific evidence for purity, consistency and health claims with proper labeling of active ingredients (Government of Canada, 2004e). An integrated approach combining micropropagation with controlled environment greenhouse propagation will produce plants that are free of contamination (heavy metals, microbes, weeds or organic pollutants) and contain standardized biochemical profiles correlated to medicinal efficacy. The challenges in commercialization of holy basil in Canada include: (a) the limited availability of plants, (b) the lack of efficient methods for controlled environment production, (c) incomplete
chemical profiling, and (d) the lack of varieties suitable for indoor use, home gardens or fresh produce. The current study was designed to address the issues associated with micropropagation and greenhouse production of holy basil to develop a model for introducing novel medicinal herbs in Canada.

1.11 Hypothesis:
Three main hypotheses of this study are:

1. An efficient system of propagation of holy basil will be developed using optimized media and successful transfer to greenhouse
2. Screening of *in vitro* raised populations will facilitate selection of lines with high antioxidant potential and optimum growth and transplant efficiency
3. Plants with high antioxidant potential will have variable growth behaviours in greenhouse conditions when compared to accessions with low antioxidant activity

1.12 Objectives:

In this thesis there are three major components for developing an integrated system for the production of holy basil with additional specific objectives.

1. To develop a micropropagation system for holy basil
   a. Determine the role of hormones in developing a micropropagation system for holy basil
   b. Investigate the use of additional media additives not previously used in holy basil to improve the established propagation system. These additives include: alternative gelling agents, activated charcoal, liquid media
systems, the phenylpropanoid pathway inhibitors such as AIP, and use of a pH buffer

2. To explore the possibility of identifying an elite plant based on antioxidant values and further characterization of selected plant.
   a. Identify an elite plant out of a population based on DPPH antioxidant scavenging assay.
   b. Further characterize the elite plant based on tissue samples (leaf, stem, and root) and assess performance in field, greenhouse and \textit{in vitro} growth conditions.
   c. Analyze the elite plant for the medicinally significant neurotransmitters, GABA, melatonin and serotonin.

3. To characterize the morphology of the selected plant with high antioxidant activity for commercial production
   a. Conduct a growth analysis in greenhouse conditions over time to see any variation in the growth behaviour of the elite plant compared to an average population
   b. Characterize the trichomes on the leaf and petiole surface between greenhouse and field grown plants
2.0 Chapter 2: Establishment of an Efficient Micropropagation system for Holy Basil (*Ocimum sanctum* L.)

Abstract:

An efficient method of micropropagation for holy basil (*Ocimum sanctum* L.) has been established from greenhouse grown nodal explants. Sterile shoot cultures were initiated on MS basal medium with low instances of contamination (4.7%). BA was most effective among the various cytokinins evaluated for shoot development and proliferation on semi-solid medium. MS medium supplemented with 1.1 µM BA in combination with 0.3 µM GA₃ was found to be optimal for shoot elongation and multiplication. Rooting was increased by the addition of auxin and activated charcoal. IBA at concentrations of 0.5 µM with 0.6 % activated charcoal significantly increased rooting percentage, root length, and root number. Rooted plantlets were successfully transferred to greenhouse conditions with an 83% survival rate. These results suggest that large scale multiplication of holy basil is possible using micropropagation.

2.1 Introduction:

Holy basil (*Ocimum sanctum* L.) is a member of the Lamiaceae family native to South Asia and North Africa. As the name suggests, it is sacred in these regions, and is used to treat a range of ailments such as fevers, chronic disease, digestive issues, and viral, fungal or bacterial infections, along with many other health problems (Engels and Brinckmann, 2013). The capability of holy basil to fight many diseases is due to its antimicrobial, antioxidant, nervine and stimulant properties (Das and Vasudevan, 2006; Singh et al., 2012; Engels and Brinckmann, 2013).
For medicinal applications, *Ocimum sanctum* (syn. *O. tenuiflorum*) is often used as a whole plant preparation or just leaf tissue (Kumar et al., 2013). Typical methods of preparation include steeping leaves or consumption of raw plant material. These methods are described in a variety of ancient medical texts such as Ayurveda, Unani and Siddha, which all describe the numerous benefits of holy basil for both treatment and prevention of diseases (Gupta et al., 2002).

The popularity of holy basil and other traditional medicinal plants is growing consistently in the Western world with an increasing interest in alternative medicine for acute and chronic illnesses (Government of Canada, 2009). In 2007 in the US, patients spent $33.9 billion on alternative medicine (Nahin et al., 2009). People and organizations are searching for new methods and are going towards alternative and preventative medicine to improve daily health and reduce long-term medical costs. With 70% of Canadians trying NHPs and the increase in ethnicities in Canada (Government of Canada, 2009), there is a strong motivation for introducing traditional medicinal plants into Canadian marketplace.

Within Canada, to be able to introduce holy basil or any other natural health product (NHP), a variety of regulations need to be met before it can be approved as safe for the general population. These regulations are outlined in the document SOR/2003-196 in the Food and Drug Act of Health Canada (Government of Canada, Health Canada, 2013). Briefly, the finalized product requires a product licence to be sold in Canada. To obtain this licence the product needs to be approved via an application process requiring details on the active ingredient content and needs to meet good manufacturing procedures (GMP), ensuring no contamination and appropriate,
allowable levels of pesticides present, amongst other regulations. To achieve this approval, chemical composition standards are required which can be challenging with NHPs as they are highly variable. This variability in active ingredients does not allow for consistency of medicinal products. Due to this variation in chemistry of plants, the development of a standardized protocol for clonal propagation would be ideal for introduction of holy basil in Canada.

To achieve high quality standards for holy basil and overcome restrictions with establishing a Canadian NHP market, an integrated propagation system needs to be developed. An efficient propagation system while maintaining GMP procedures will also ensure high standards of safety and can overcome the other issues with producing a NHP, such as quality, consistency, and purity of the product. Alternatives to traditional methods of cultivation through seed can ensure these regulations are met, while overcoming issues such as low germination rates and poor vegetative propagation.

Micropropagation is a sterile culture system within indoor growth facilities. Asexual clonal propagation of elite plants can be utilized to maintain genetically uniform stock material. For medicinal plants, this method of propagation can be highly beneficial because one can identify plants with high levels of active compounds and clone them through tissue culture, further maximizing the efficacy of the product (Murch and Saxena, 2006). Other benefits of this method are: (1) many plants can be kept in small systems, (2) optimization of growth conditions can ensure high multiplication rates, and (3) the sterile environment maintains elite plants with no cross contamination of chemical and biological pollutants (Rout et al., 2000). The problems that are possible with micropropagation may occur during establishment of a proper culture system from
the initiation of sterile populations, multiplication, rooting, and the final transfer into greenhouse environments. Within these stages, proper hormones, micro- and macro-nutrients, as well as other inputs, need to be optimized to ensure optimal growth and high yield of desirable material (George et al., 2008).

Micropropagation can be accomplished through node culture and is frequently used in clonal propagation systems, as fewer instances of callus and adventitious shoots develop (George et al., 2008). For medicinal plants, this method is ideal as it will improve genetic and phenotypic stability. The use of this system will be ideal for holy basil to clone elite plants for medicinal activity and desirable physiological traits such as large yields of leaf tissue.

Currently, research in holy basil micropropagation has been limited. Studies have investigated protocols for holy basil, but a consistent procedure from establishment of sterile culture to greenhouse production has not been described. Establishment of culture has been accomplished using various explant material including: inflorescence (Singh and Sehgal, 1999), leaf tissue (Shilpa et al., 2009; Banu and Bari, 2007; Xiong Lim et al., 2009), and axillary or nodal segments (Mandal et al., 2000; Tyub and Kamili, 2009; Gogoi and Kumaria, 2011; Girija et al., 2006; Banu and Bari, 2007) with high rates of success.

Studies investigating holy basil for multiplication and shoot production from source material are divided on the optimal hormonal concentration and combination. These studies have shown that BA is a suitable cytokinin for shoot production (Banu and Bari, 2007; Mandal et al., 2000; Pattnaik and Chand, 1996; Shahzad and Siddiqui,
2000; Singh and Sehgal, 1999; Tyub and Kamili, 2008). However, concentrations in these articles range from 0.88 - 22µM of BA and some describe the use of BA in combination with other growth regulators such as IAA (Singh and Sehgal, 1999), GA₃ (gibberellic acid) (Pattnaik and Chand, 1996), or NAA (Shahzad and Siddiqui, 2000) for improving shoot production. In addition, some researchers found that increased production of shoots did not require the addition of BA and the combination of NAA with KN was best for shoot multiplication (Gogoi and Kumaria, 2011).

Although high variation of shoot production is seen with holy basil, rooting of sterile shoots is described as being successful with 90-98 % efficiency (Banu and Bari, 2007; Pattnaik and Chand, 1996). Overall, the use of NAA at various concentrations is described in different studies as being ideal for root production (Shilpa et al., 2009; Banu and Bari, 2007; Gogoi and Kumaria, 2011; Shahzad and Siddiqui, 2000; Pattnaik and Chand, 1996). Some studies also describe the use of other auxins such as IAA and IBA to increase root production in vitro (Banu and Bari, 2007; Pattnaik and Chand, 1996). Once the establishment of root systems is completed, plants can be acclimatized and transferred into greenhouse systems for mass production. Literature describes survival rates ranging from 70-85% after transfer to the greenhouse (Gogoi and Kumaria, 2011; Pattnaik and Chand, 1996; Singh and Sehgal, 1999).

As seen in the literature, progress has been made in determining micropropagation methods; however, an optimized protocol has not been established and the genotypic specificity is not known. Although, the literature is descriptive, an optimal ratio of hormones and overcoming issues commonly found within tissue culture processes have not been described. Thus, a complete, reproducible process for
production of chemically defined plant population is not available for commercializing holy basil. This study was designed to develop a comprehensive protocol of propagation for holy basil, from culture establishment to greenhouse transplant using various hormones, additives and methods. Development of a reproducible and efficient system of micropropagation will also be useful for discovery of novel metabolites from this unique medicinal plant.

2.2 Material and Methods

2.2.1 Germination of Seeds:

Seeds were acquired from Richter’s Seed Company (Goodwood, ON), and were surface sterilized using a 15% (v/v) solution of commercial bleach (Clorox®, The Clorox Company; 5.4% sodium hypochlorite) for four minutes while being agitated. Seeds were then washed three times with sterilized distilled water for three minutes each. Sterile seeds were used for experimentation using GA₃ and red light to optimize germination rate.

Gibberellic Acid

Sterile seeds were either pre-treated with GA₃ or plated onto Petri dishes containing a semisolid medium supplemented with GA₃. For the pre-treatment, seeds were soaked in a GA₃ solution at four levels (0, 29, 145 and 290 µM) for 24 hours. Afterwards 20 seeds per dish were randomly plated onto a semisolid medium containing MS salts, 2% sucrose (w/v) and B5 vitamins and solidified with 2.2g/L Phytagel (Sigma Aldrich). Each treatment was replicated five times for a total of 100 seeds per treatment.

For the GA₃ treatment in culture, the media containing MS salts, 2% sucrose (w/v) and B5 vitamins were supplemented with 0, 0.29, 2.9 or 14.5 µM concentration of
GA₃ and solidified with 2.2g/L Phytagel (Sigma Aldrich, Oakville, Canada). For each treatment, five plates were used with approximately 20 seeds per plate. For both experimental procedures seeds were placed in dark conditions in growth chamber rooms at 24±2°C, and after four and six days the germination rate was measured for each plate.

Red light treatment

Sterilized seeds were plated on Petri plates containing MS salts, 2% sucrose, B5 vitamins and 2.2g/L Phytagel. Growth chambers producing red light (660nm) were used to provide red light time lengths of 0, 30, 60, 360 and 1440 minutes to seeds with 20 plates per treatment. Germination rates after red light treatment was measured after four and six days.

2.2.2 Plant material and culture establishment

In vitro shoot cultures were established using two year old plants grown in greenhouse conditions from a single source plant in the greenhouse. Approximately 40 fresh nodal explants with single shoot buds were collected and cleaned under running tap water for 60 min. Surface sterilization was conducted by dipping the explants in 70% (v/v) ethanol for one minute followed by three minutes of rinsing in sterile deionized water. Shoot buds were further surface disinfected in 15% (v/v) solution of commercial bleach (as described for seed sterilization) for 10 min with intermittent agitation. Finally, the explants were rinsed three times with sterile distilled water, each lasting four minutes. Buds were then excised at the basal end and cultured in culture tubes each containing 10 mL of semisolid (supplemented with 2.2 g/L Phytagel) or liquid basal MS medium with B5 vitamins supplemented with 3% (w/v) sucrose. The cultures were
maintained in a growth room at 24±2°C under a 16h photoperiod (40 µmolm⁻²s⁻¹)
provided by cool white fluorescent lamps (Osram Sylvania Ltd., Mississauga, Ontario).

For experiments on various concentrations of cytokinins, established shoots were
removed from sterile culture and were excised from the basal end after four weeks.
Shoots were then transferred into sterilized test tubes with about 10-15 mL of semi-solid
media containing different cytokinins: BA, KN or TDZ at concentrations of 0.5, 1.0 or 2.0
µM, based on previous references, with 10 replications of each treatment. After four
weeks of maintenance in standard growth room conditions, cultures were assessed for
shoot height and number of shoots per explant.

Due to the strong positive response of BA, it was used for further experiments to
optimize the proliferation medium. After four weeks, shoots developed on MS basal
medium were excised from the basal end and transferred into Magenta GA7 culture
vessels (PhytoTechnology, Kansas, USA) each containing 50 ml of semi-solid basal MS
medium supplemented with BA (0.0 µM, 1.1 µM, 2.2 µM and 5.5 µM). Four shoots with
2-3 internodes were placed in each magenta box with three replications. Plants were
measured after four weeks in culture for shoot height and number of shoots per explant.

The antiauxins TIBA (2,3,5-triiodobenzoic acid) and PCIB (p-
chlorophenoxyisobutyric acid) were studied as described above for BA. Using 50 mL of
semi-solid MS basal medium with TIBA or PCIB at concentrations of 0 1.0, 2.0 and 5.0
µM, four shoots were added in each magenta box with three replications each. Plants
were measured after four weeks in culture for shoot height, number of internodes and
shoots per explant.
2.2.3 Rooting and Plantlet Development:

Shoots were obtained from sterile tissue culture with at least three nodes. The apical shoot tips were excised from shoot cultures and cultured in Magenta GA7 culture vessels (PhytoTechnology, Kansas, USA) with four explants per box. The boxes contained 50 mL of semisolid media composed of MS salts, B5 vitamins, 3% (w/v) sucrose and 2.2% (w/v) Phytagel (Sigma-Aldrich, Oakville, Canada) and supplemented with four concentrations of IBA (0.0 µM, 0.5 µM, 2.5 µM, 5.0 µM) with or without the addition of activated charcoal at 0.6% (w/v). The cultures were maintained in a growth room at 24±2°C under a 16h photoperiod (40 µmolm⁻²s⁻¹) provided by cool white fluorescent lamps (Osram Sylvania Ltd., Mississauga, Ontario). After four weeks in culture the plants were removed from the media and observed for root length, root number and rooting percentage per treatment.

2.2.4 Greenhouse Acclimatization:

Micropropagated shoots were cultured on the optimized rooting medium (basal MS medium supplemented with 0.5 µM IBA and 0.6% activated charcoal) to obtain a sufficiently large population for acclimatization trials. In vitro rooted shoots were removed from the culture medium and washed gently under running tap water. A total of 12 plants with three replications were transplanted into 18-cell trays filled with Sunshine professional growing mix (Sun Gro Horticulture, Vancouver, British Columbia). All trays were placed in a mist bed for a week and later transferred into the greenhouse. The percentage of survival was recorded after three weeks of greenhouse transplant.
2.2.5 Statistical analysis:

All the experiments were conducted using a completely randomized design and analysis of variance was conducted using JMP version 9.0.2 for Windows (SAS Institute Inc. Cary, NC). Means were compared using Tukey’s test (P< 0.05). The data are presented as means ± standard error of means.

2.3 Results:

2.3.1 Germination of Seed:

Seeds were sterilized with low occurrence of contamination. A pre-treatment of GA$_3$ or seeds plated with GA$_3$, did not significantly increase germination percentage for all treatments when compared to the water control (Figures 2.1, 2.2A, B). Plates containing 2.9 µM had the highest seed germination percentage at 3.81%, and 0.29 µM with a rate of 3.03%, while the control had a 2.0% germination rate (Figure 2.2B). Use of red light treatment on seeds also did not show any significant differences in germination percentage compared to the control (Figure 2.2C).
Figure 2.1 *Ocimum sanctum* L. seeds plated on different treatments for germination rates; (A) seeds in basal medium after four days (B) seeds on medium with GA$_3$ after six days.
Figure 2.2 Germination observations of holy basil in both GA$_3$ and red light treatments at four and six days after plating (A) Germination percentage of seeds pre-treated with GA$_3$ for 24 hours. (B) Germination percentage of seeds plated on medium containing concentrations of GA$_3$. (C) Germination percentage of seeds treated with red light for different time lengths. Columns with same letter indicate no significant difference (P<0.05)
2.3.2 Culture initiation and establishment:

Shoot bud development was observed on Murashige and Skoog (MS) (Murashige and Skoog, 1962) culture medium with 91.05% success rate, while nearly 4.7% buds were found to be contaminated. The addition of cytokinins (BA) to the medium was not essential for the successful establishment of shoot cultures and it generally reduced the response rate of the explants.

After four weeks, shoots with 2-3 internodes were transferred onto medium supplemented with different cytokinins. Among the cytokinins tested, BA was the most effective for shoot proliferation followed by control basal medium. Treatment with BA (1.0 µM) showed significantly higher numbers of shoots (2.5) compared to the control (Figure 2.3). Treatments with BA (2.0 µM) and KN (1.5 µM) were significantly better than the control, but only had 1.6 and 1.5 shoots per explant, respectively. Height was not significantly different than the control for the BA treatments; however KN and TDZ treatments had a significant decrease in height compared to the control (Figure 2.4).

The MS basal medium supplemented with BA (1.1 µM) and GA₃ (0.3 µM) was the most effective treatment compared with other levels (Figures 2.5, 2.6, 2.7). Culture medium with and without GA₃ was not significantly different, but a visual improvement in plant health was observed with the addition of GA₃. All concentrations of BA showed an increase in the number of shoots compared to the control medium, but was highest in the concentration of BA at 1.1 µM, with 2.5 shoots per explant, significantly different from the control (Figure 2.5). The maximum number of internodes (5.8) was observed with media containing BA at 1.1 µM (Figure 2.6), significantly higher than the control.
BA at higher concentrations (4.4 µM) resulted in fewer internodes (3.5) compared to the other concentrations of BA. Shoot height had a similar pattern to the number of internodes, with BA at levels of 1.1 µM being the tallest at 56 mm (Figure 2.7). This was not significantly different than the control with an average height of 53 mm. The shoots in control and BA 1.1 µM were significantly taller than those grown on 2.2 µM (45 mm) and 4.4 µM (29 mm) BA. Overall, a higher multiplication rate and more internodes were obtained at 1.1 µM BA (Figure 2.8), while shoot length was similar to that of the shoots developed in the control. Higher levels of BA adversely affected shoot proliferation and produced shoots with smaller leaves and shorter internodes.
Figure 2.3 Average number of shoots produced out of 10 replications of holy basil. Nodal cultures were measured after four weeks in culture of three different cytokinin treatments (BA, KN or TDZ) compared to the control (MS). Columns with same letter indicate no significant difference (P<0.05)

Figure 2.4 Average shoot height (mm) of holy basil nodal cultures with 10 replications of each treatment after four weeks in culture of three different cytokinin treatments (BA, KN or TDZ) compared to the control (MS). Columns with same letter indicate no significant difference (P<0.05)
Figure 2.5 Average number of shoots on different media containing BA (0 – 4.4 µM) with GA₃ (0.3 µM). Nodal segments were measured after four weeks with 10 replications per treatment. Columns with same letter indicate no significant difference (P<0.05)

Figure 2.6 Average number of internodes after four weeks in culture in BA (0 – 4.4 µM) and GA₃ (0.3 µM). Replications of 10 nodal segments were used for each treatment. Columns with same letter indicate no significant difference (P<0.05)
Figure 2.7 Average shoot length (mm) after four weeks in culture in BA (0 – 4.4 μM) and GA₃ (0.3 μM). Replications of 10 nodal segments were used for each treatment. Columns with same letter indicate no significant difference (P<0.05)
Figure 2.8 Photographs demonstrating shoot multiplication with the use of cytokinin treatment with BA after four weeks in culture (A) *Ocimum sanctum* L. single shoot formation in basal medium (B - D) *Ocimum sanctum* L. shoot multiplication on BA 1.1 µM with GA3 0.3 µM media.
2.3.3 Effect of antiauxin on shoot multiplication:

The addition of antiauxin to growth medium had no significant effects compared to the control for the number of shoots, except for the treatment with TIBA at 5 µM which was significantly less effective than the control (Figure 2.9). Number of internodes showed significant increases with all PCIB treatments, and in TIBA at 2 and 5 µM. Average height significantly decreased with the use of PCIB at 5 µM and TIBA at 2 and 5 µM. Delayed shoot development and poor growth were observed in the culture medium with TIBA at all concentrations compared to medium with PCIB. In general, shoots were observed to have a thick stem and shorter internodes with higher rooting in the medium treated with PCIB at all concentrations when compared to the control treatment. Apical explants from the shoots developed on the medium containing PCIB, showed only 40% shoot development when transferred to the basal medium.
Figure 2.9 Effect on average number of shoots and internodes on nodal segments (n=12) supplemented with antiauxin PCIB and TIBA (1, 2, 5 µM) treatments after four weeks. Columns with same letter indicate no significant difference (P<0.05)

Figure 2.10 Average shoot height of nodal segments supplemented with PCIB and TIBA antiauxins at 1, 2 and 5 µM (n = 12) after four weeks. Columns with same letter indicate no significant difference (P<0.05)
2.3.4 Rooting and plantlet development

Shoot explants with 2-3 nodes were excised from 4-6 week old cultures and transferred to media containing auxin with and without activated charcoal (Figure 2.11). The highest number of roots (10.3), root length (40.2mm) and percentage of rooting (88.9%) were obtained on medium supplemented with 0.5 µM IBA with and without 0.6% activated charcoal (Figures 2.10, 2.11, 2.12). These values were all significantly greater than the control with and without activated charcoal. Rooting percentage using IBA at concentrations of 2.5 and 5.0 µM with and without activated charcoal were not significantly different than the control (Figure 2.12). The average number of roots on plants cultured with 2.5 µM IBA was not significantly different than the control. The use of 5.0 µM IBA however, was significantly less effective than the control (Figure 2.13). This trend was also observed with the root length (Figure 2.14). Overall, medium containing IBA at 0.5 µM with the combination of 0.6% activated charcoal was found to be the most effective to stimulate the development of an improved rooting system when compared to the control.

2.3.5 Greenhouse acclimatization:

Rooted plantlets were acclimatized in the greenhouse and had an 83% survival rate after 4 weeks (Figure 2.16). Initially, rooted plantlets kept in misting bed for a week (Figure 2.15) showed a better survival rate compared to plants acclimatized in the growth chamber.
Figure 2.11 Photographs demonstrating root development from *in vitro* grown holy basil plants in IBA 0.5 µM and AC (0.6%) for four weeks (A) Root growth of microshoots in media (B) Significant root development on microshoots

Figure 2.12 Average rooting percentage of microshoots (n = 10) cultured in IBA media (0 – 5 µM) with and without activated charcoal after four weeks in culture. Columns with same letter indicate no significant difference (P<0.05)
Figure 2.13 Average root number (n = 10) after four weeks in culture in various IBA treatments (0 – 5.0 µM) with and without activated charcoal. Columns with same letter indicate no significant difference (P<0.05)

Figure 2.14 Average root length (mm) after four weeks in culture with different IBA concentrations (0 – 5.0 µM) with and without the addition of activated charcoal. Columns with same letter indicate no significant difference (P<0.05)
Figure 2.15 Holy basil microshoots after rooting in media containing 0.5 µM of IBA and 0.6% AC for 4-6 weeks, were transferred into 24 well 1" trays (A) and put into mist bed chambers for a minimum of two weeks (B) before transfer to greenhouse conditions.

Figure 2.16 Photograph of *Ocimum sanctum* L. *in vitro* grown plants successfully transferred to greenhouse for six weeks.
2.4 Discussion:

The use of micropropagation in medicinal plants is common and in the present research a protocol for propagating holy basil *in vitro* is described. Traditionally, holy basil is cultivated outdoors from seed, encouraging variation and inconsistency in the resulting population. Using the nodal method of cloning, elite plants can be multiplied and maintained in sterile environmental conditions and then introduced into greenhouse for increasing yield of leaf tissue. This method is also favourable for medicinal plants as the controlled environment allows for meeting Health Canada’s regulations regarding the production of safe NHPs.

2.4.1 Germination and Initiation:

Holy basil seeds were germinated in basal and GA₃ supplemented medium and also exposed to red-light conditions to determine optimal conditions for increasing germination. In this study the germination rates of holy basil seeds were fairly low and the use of treatments before and during germination had no significant effect on germination rates.

The use of red light for germination of plant seeds is often observed. Initially described in lettuce (Miller, 1958), the use of red light in improving seed germination is due to the interaction with photoreceptors and phytochromes, specifically phytochrome B. Activation of phytochrome B begins a transcription pathway leading to degradation of a seed germination inhibitor. In this report, no significant effect on germination was observed with timed treatments of red light. This conclusion was also seen in the study on *Ocimum tenuiflorum* seeds by Amritphale and Mall (1981), who found that red light
did not improve germination when compared to white light. As well, *Ocimum gratissimum* L. seeds required long treatments of red light before increases in germination rate were observed, suggesting that high energy over a long term is required to initiate germination.

Gibberellic acid was also investigated as a seed pre-treatment and as a supplement to growth medium to determine germination rates. GA$_3$ is known to induce germination through its ability to overcome dormancy in seeds (Kahn et al., 1957; George et al., 2008). In both types of treatments with GA$_3$, germination rates were low, with no significant differences between the seed pre-treatment and plates containing GA$_3$. The application of GA$_3$ has been used in other systems of holy basil for seed germination. Amritphale and Mall (1981) found that GA$_3$ helped to overcome dormancy with *O. sanctum* seeds. The use of GA$_3$ at 29 µM also improved germination rates in *Ocimum sanctum* seeds but the time of harvest of seeds had a greater influence on germination rate which was not improved even with hormonal treatment (Dey and Choudhari, 1984). This variation in germination of holy basil seeds as observed in several studies is likely to be a result of physiological status of seeds. Holy basil seeds develop asynchronously and display great variation in maturity. Thus the seed collected at different times may have very different profiles of endogenous hormones and other metabolites. Regardless, poor seed germination in this species further highlights the importance of a micropropagation method for stock maintenance and plant multiplication.

Two year old seed derived plants from the greenhouse were used to initiate and establish *in vitro* cultures. Nodal explants introduced into sterile environments were
highly successful with the sterilization procedure described, as low instances of contamination occurred (< 5%). This high success rate may be due to the natural anti-fungal and anti-bacterial properties of holy basil (Mondal et al., 2009; Gupta et al., 2002). Plants were initially cultured in MS basal medium, with a high degree of success (95%) which was better than that obtained in previous reports (Pattnaik and Chand, 1996) with only 35-40% bud break on hormone free medium for mature holy basil plants. In contrast, Banu and Bari (2007) have shown that a cytokinin (BA) is required to initiate bud break of holy basil. The use of MS salt based media in this experiment was highly effective and this observation is similar to previous reports on micropropagation of holy basil (Banu and Bari, 2007; Singh and Sehgal, 1999; Pattnaik and Chand, 1996; Xiong Lim et al., 2009; Tyub and Kamili, 2009; Mathew, 2011).

2.4.2 Multiplication and Development of Shoots:

In the present study, the cytokinins BA, KN and TDZ at concentrations of 0.5, 1.0 and 2.0 µM were tested for shoot development and to increase the multiplication rate. BA was the most effective cytokinin to increase the number of shoots per explants, internodes, and shoot height. BA is the most common cytokinin used for shoot development and multiplication in a range of species (Pattnaik and Chand, 1996; Mandal et al., 2000; Ault et al., 2004; Cole et al., 2007; Ault and Havens, 1999; Sahoo et al., 1997). In a study on holy basil conducted to compare BA and KN for numbers of shoot and shoot height, a significantly improved response with BA as compared to KN was observed (Banu and Bari, 2007). BA has also been described as being optimal for micropropagation, specifically for shoot induction from various explants of *O. basilicum* (Sahoo et al., 1997), *O. americanum* and *O. gratissimum* (Pattnaik and Chand, 1996).
Micropropagation of other medicinal plants such as *Atropa belladonna* (Benjamin et al., 1987) and *Clerodendrum colebrookianum* (Mao et al., 1995) has also been achieved successfully using BA. In holy basil, a lower level of KN (2.32 µM) has also been used as the only cytokinin source for inducing shoot development (Gogoi and Kumaria, 2011).

The efficacy of cytokinin is generally variable depending on species in regard to optimal concentration and type (Mao et al., 1995; Singh and Tiwari, 2012; Gaspar et al., 1996) due to endogenous hormone levels, type of explant and the genetic variability among genotypes and species. Because of the positive effect of BA observed in this study, it was used exclusively for all further experiments on shoot proliferation.

After four weeks, shoots were maintained in culture medium and shoots with 2-3 internodes were used for further experiments to optimize multiplication rates and increase shoot proliferation. The results of the investigation to improve shoot multiplication showed that holy basil shoot cultures were well maintained on MS basal medium with the cytokinin BA at 1.1 µM and gibberellic acid (GA\textsubscript{3}) at 0.3 µM. With optimized medium it was possible to obtain shoot cultures which had an average shoot length of 56.3 mm, 5.8 internodes per explant and 2.5 shoots per explant. In a similar study, holy basil axillary buds growing in MS medium containing BA (1.0 µM) responded best for branching and shoot length, and shoot response reduced significantly with an increase in concentration of BA (Tyub and Kamili, 2009). Banu and Bari (2007) used a range of concentrations of BA and found a maximum shoot number and length with a low concentration of BA (0.88 µM). In comparison, some studies found higher levels
(4.4 µM) of BA were required for optimum proliferation from axillary buds and as many as 12 – 14 shoots per explant were obtained (Mandal et al., 2000).

The combination of cytokinin and gibberellic acid is known to improve shoot growth in some species (Shukla et al., 2012; Pattnaik and Chand, 1996; Engelke et al., 1973). Although the addition of GA₃ did not produce significant differences in the proliferation rate in this study, the development and growth of shoots seemed visually superior compared to the culture raised with BA alone. Therefore, the use of the combination of BA and GA₃ in the culture medium was continued for further investigation. Pattnaik and Chand (1996) also reported similar beneficial effects of the use of BA (4.9 µM) and GA₃ (1.45 µM) in combination for shoot multiplication of holy basil. The use of GA₃ is common for shoot induction from adventitious bud explants in other basil species such as *O. basilicum* (Sahoo et al., 1997), *O. americanum* and *O. gratissimum* (Pattnaik and Chand, 1996). This positive effect of GA₃ is thought to be a result of the stimulation of growth and elongation of cells (Rout et al., 2000). However, due to its potency GA₃ is better used at lower concentrations as it can be have both positive and negative influence on *in vitro* shoot production (Thiyagarajan and Venkatachalam, 2013; Purkayastha et al., 2010; Singh and Tiwari, 2012).

The effects of antiauxins TIBA and PCIB were investigated to determine if they improved shoot multiplication and shoot height. Observations of these experiments indicated that they inhibited shoot growth. The cytokinin to auxin ratio must be carefully maintained in plants to direct desired growth and development (Skoog and Miller, 1957). Imbalance of plant growth regulators and nutrients has the capacity to alter tissue growth and multiplication. The inhibition of shoot growth by the addition of antiauxins
may indicate that natural auxin levels of tissues were in balance when explants were
grown in the presence of a cytokinin (BA). Addition of auxin inhibitors of transport (TIBA)
or biosynthesis (PCIB) is likely to modulate endogenous profiles of both auxin and
cytokinins. Similar effects on endogenous phytohormones may occur with the addition
of exogenous auxin or cytokinin. Previous studies on holy basil shoot development have
shown that the use of an exogenously supplied auxin (IAA, IBA, NAA) in addition to a
cytokinin (BA, KN) can inhibit or promote shoot growth (Gogoi and Kumaria, 2011;
Shahzad and Siddiqui, 2000; Singh and Sehgal, 1999), indicating the regulation of
morphogenesis mediated by endogenous factors.

Morphogenetic responses in vitro are influenced by the levels of endogenous
phytohormones and nutrients (Skoog and Miller, 1957), which in turn are influenced by
the physiological and genetic characteristics of the source plants. The variation in
environmental conditions of the donor plants coupled with stresses on in vitro culture
can significantly alter the balance of endogenous growth regulators. Thus, the observed
differences in shoot development in cultures of holy basil in various studies may result
from the variation in source of seeds, genotypes, and conditions of growth and
manipulation of plants and explants. The beneficial or deleterious effects of adding
exogenous antiauxin to the medium on shoot development may also be ascribed to
altered balance of auxins and cytokinins as observed in various culture systems (Shukla
et al., 2012).
2.4.3 Development of Roots In Vitro:

The use of auxin to initiate rooting is frequent in micropropagation techniques. Exogenously applied auxin and cytokinin ratios can influence organ development in vitro, which can also be affected by endogenous hormonal levels. A ratio of auxin to cytokinin at approximately 100 is thought to favour root formation within in vitro cultures (Rout et al., 2000).

Nearly 40% of the cultured shoots of holy basil formed roots in vitro on basal medium, but the frequency of rooting increased to 89% with the addition of IBA at 0.5 µM along with 0.6% activated charcoal. Earlier studies have also shown that rooting of micropropagated holy basil can be accomplished on basal or auxin supplemented medium (Pattnaik and Chand, 1996; Banu and Bari, 2007; Gogoi and Kumaria, 2011). Banu and Bari (2007) reported induction of roots on 80% of shoots with a low concentration of IBA (0.49 µM). However, in the method described in the current study, an increase in number of roots per culture was observed compared to Banu and Bari (2007) with only 3.75 roots per culture with the use of IBA. Pattnaik and Chand (1996) found that for holy basil shoots, IBA at higher concentrations (2.49 and 4.9 µM) was required to improve root formation to 76 - 89%.

In the present study, the use of hormone free medium produced roots, but the incidence was significantly lower than that obtained for shoots cultured with activated charcoal and IBA. In several other medicinal plants, the use of basal medium without an added auxin has been described for inducing roots (Mao et al., 1995; Santos et al., 1990; Saxena et al., 1997). Singh and Sehgal (1999) also found that in cultures of holy
basil the use of hormone free media produced significant amounts of roots *in vitro*. In contrast, Pattnaik and Chand (1996) observed that the use of a hormone free medium resulted in no rooting in any of the shoots of holy basil. Together, these results suggest that the shoots that require auxin treatment to root have low endogenous auxin or the endogenous cytokinins are too high causing an imbalance of auxins available for root induction. A decrease in the rooting percentages of holy basil shoots was observed with the use of high concentrations of IBA. Similar results were reported for *Aegle marmelos* (Hossain et al., 1993). This inhibition of rooting can again be explained on the basis of excess auxin resulting from addition of high exogenous IBA which is likely to cause an imbalance in the cytokinin to auxin ratio, leading to improper rates of cell division, elongation, and further root development.

The use of activated charcoal (AC) for *in vitro* culture systems of holy basil has not been previously described. In this study, an improvement in root percentage, length and number was observed with the addition of 0.6% (w/v) activated charcoal in combination with 0.5 µM IBA when compared to the basal control. This improvement is thought to be due to the adsorbing ability of the charcoal. AC adsorbs chemicals and plant growth regulators within the media and those released by the tissue. AC also provides a dark growth environment in culture and also releases substances into the media, which may have beneficial effects for *in vitro* culture (Pan and Staden, 1998). Although not previously described in holy basil the use of AC *in vitro* for rooting has been effective in other medicinal plants such as *Citrullus colocynthis* (Meena et al., 2010); *Pogostemon cablin* (Kumaraswamy and Anuradha, 2010), *Salvia africana-lutea* (Makunga and Staden, 2007).
As stated earlier, plant morphogenesis is regulated primarily by interaction of endogenous and exogenous plant growth regulators, nutrients, and various stresses (Skoog and Miller, 1957; Cassells, 2005; Murch and Saxena, 2005). The inclusion of AC in growth medium can cause significant changes in phytohormone and nutrient profile of the growing tissue via adsorption of growth regulators making them unavailable. Alternately, AC can influence growth pattern by causing osmotic changes and influencing light conditions in immediate growth environment.

2.4.4 Acclimatization of Plants in Greenhouse:

Plantlets after rooting at 4 weeks were transferred to mist beds to acclimatize over an additional period of 2 weeks. They were then transferred to greenhouse conditions with an 83% survival rate. The use of high humidity conditions is not uncommon for acclimatization of plants before introduction into greenhouses. This is due to the requirement for the plants to transfer energy production from a heterotrophic to autotrophic mode, generating sugars through photosynthesis and not obtaining them from the culture medium. Increasing humidity assists in this by maintaining the conditions found in vitro and allowing plants to slowly adapt to the change in nutrient production and water loss through transpiration. This necessity of high humidity for holy basil is also reported by numerous authors (Pattnaik and Chand, 1996; Gogoi and Kumaria, 2011; Singh and Sehgal, 1999). Although the soil composition is slightly different for each report, the percentage of survival of holy basil described here falls within the previously reported range of 70-85%.
2.5 Conclusion:

An efficient protocol for the micropropagation of holy basil was developed. This method describes the use of nodal segments to initiate cultures, followed by shoot growth and multiplication, rooting and finally the acclimatization before introduction into the greenhouse for large-scale production (Figure 2.17). The use of the cytokinin BA (1.1 µM) in combination with GA\(_3\) (0.3 µM) was optimum for improving shoot development. Rooting medium containing IBA at 0.5 µM along with activated charcoal at concentrations of 0.6% (w/v) had the highest rooting percentage, and the slow acclimatization of holy basil in high humidity environments before transfer to greenhouse conditions ensured a high survival rate of plants. Improvement of the multiplication media could be explored further to increase the number of shoots per explant. Additionally, plants in long term culture demonstrated browning and destabilization of the media decreasing efficiency for a large scale system. Continued experimentation into optimization of the multiplication media to increase multiplication rates and decrease subculturing frequency would be beneficial for holy basil production.
Figure 2.17 Summary of the multistage process of micropropagation of holy basil from establishment to transfer to greenhouse conditions
Chapter 3: Multiplication of Holy Basil (*Ocimum sanctum*) in vitro: Improving the multiplication rate through the addition of media supplements

**Abstract:**

Experiments were conducted to investigate the effect of the addition of different media supplements and physical states including the use of liquid culture systems, evaluation of different gelling agents, application of pH buffers, and addition of activated charcoal and 2-aminoindane-2-phosphonic acid (AIP) to culture media. Liquid culture, agar as a gelling agent, and pH buffers, all had detrimental effects on the plant growth and survival. The use of activated charcoal at 0.6% improved multiplication of the holy basil plantlets to almost 4 shoots per explant. Addition of AIP, a phenylalanine ammonia lyase (PAL) inhibitor to the medium at 2.0 µM resulted in better plant growth and multiplication rate (6.3), decreased leaf browning, and reduced additional issues with the media. Supplementation of activated charcoal or AIP to the medium improved plant growth and multiplication rate while reducing the frequency of subculturing. The use of activated charcoal and AIP was determined to be the most efficient treatment for improving micropropagation.

**3.1 Introduction:**

Holy basil (*Ocimum sanctum*) is a culturally and medicinally important plant traditionally used throughout South Asia and Northern Africa. While it is traditionally propagated by seed, this approach leads to high levels of chemical diversity and inconsistent herbal products (Engels and Brinckmann, 2013; Tyub and Kamili, 2009). To reduce this variability and produce more consistent and higher quality products, several
authors have proposed the use of micropropagation to mass produce genetically uniform populations (Rout et al., 2000; Kumaraswamy and Anuradha, 2010; Tyub and Kamili, 2009). Experiments reported in Chapter 1 showed that the micropropagation protocol produced 2.5 shoots per explant. However, destabilization of the media necessitated frequent subculturing and reduced the efficiency of the protocol precluding its commercial application. Therefore, improving both the efficiency and the multiplication rate was explored in subsequent studies reported in this chapter.

Previous research on holy basil in tissue culture has not compared the gelling agents used in culture media. All of the previous research used various concentrations of agar as solidifying agent (Singh and Sehgal, 1999; Pattnaik and Chand, 1996; Banu and Bari, 2007; Shahzad and Siddiqui, 2000; Shilpa et al., 2009). Plant tissue culture media used in semi-solid production systems can be solidified using a variety of gelling agents. In the case of holy basil in this study, the medium was solidified using gellan gum, a bacterial polysaccharide that solidifies in the presence of cations forming polymers after boiling (George et al., 2008; Giavasis et al., 2000) and compared to the effectiveness of agar. Gellan gum is an effective gelling agent for a variety of species (Ishii et al., 1998; Anders et al., 1988; George et al., 2008), but is known to be more sensitive to pH levels than other agents such as agar (George et al., 2008; Giavasis et al., 2000), a gelling agent extracted from seaweed, either *Gellidium* and *Gracillaria* (Phytotechnology, 2011).

Liquid culture systems offer unique benefits for large scale production as they can increase growth rates and production of secondary metabolites (Paek et al., 2005). These systems also provide the ability to reduce labour inputs and costs when
compared to a semi-solid large scale production system (Paek et al., 2005). Liquid bioreactor systems have been used previously with high success in other medicinal plants to improve multiplication and biosynthesis of secondary metabolites (Sandal et al., 2001; Kintzios et al., 2004; Paek et al., 2005). However, the use of liquid medium can also lead to some negative effects on plant growth due to low levels of oxygen and resulting hyperhydricity of plant tissues (George et al., 2008). (CHECK all refs)

While the pH of culture media is generally adjusted to an ideal range for the species, this is generally done prior to adding the gelling agent and autoclaving the media (Singh and Sehgal, 1999; Tyub and Kamili, 2009; Kumaraswamy and Anuradha, 2010). Impurities in the gelling agents and thermal degradation products during sterilization can alter the pH (Owen et al., 1991). More importantly, many plant species produce exudates, including phenolic acids that can change the pH of the media over time (George et al., 2008). To address this issue, buffers are sometimes added to help stabilize the pH. Another approach that has not been previously explored in holy basil is the addition of activated charcoal in growth media. Activated charcoal particles have a large, oxidized surface area (Thomas, 2008) with large pores that contribute to the adsorbent behaviour. The use of activated charcoal is common in in vitro protocols to decrease browning, stabilize pH, adsorb negative chemicals produced by the medium or plant and provide a dark environment for in vitro cultures (Thomas, 2008; George et al., 2008; Pan and Staden, 1998). While these techniques have not been evaluated to culture holy basil, they have been described to improve plant multiplication in other species (Tascan and Adelberg, 2010; Moraes et al., 2003)
Holy basil plants often displayed leaf browning in multiplication culture media. Oxidative browning is a common problem in plant tissue culture and is a result of the release and oxidation of phenolic compounds (Hisaminato et al., 2001; Krishna et al., 2008; Rout et al., 2000). It is possible that the compounds that lead to tissue browning, including phenolic acids, are also responsible for the need of frequent subculturings. Recently, 2-aminoindane-2-phosphonic acid (AIP), a competitive inhibitor of the phenylalanine ammonia lyase enzyme, has been used to reduce tissue browning in plant tissue culture (Hisaminato et al., 2001; Jones and Saxena, 2013). This approach works by inhibiting the biosynthesis and subsequent oxidation of phenolic compounds (Zoń and Amrhein, 1992; Appert et al., 2003).

The objectives of this series of experiments were to compare the different culture additives and media states not previously explored in holy basil to improve multiplication rate and decrease the need for frequent subculturings. To this end, the following approaches were evaluated; a) liquid vs. solid culture systems, b) comparison of gelling agents, c) the use of a pH buffer, d) incorporation of activated carbon, e) addition of AIP to the culture medium.

3.2 Materials and Methods:

3.2.1 General Information:

In all of the following experiments, plants were derived from seeds obtained from a market in Meerut City, UP, India. Plantlets were maintained in vitro using the medium as previously described (Chapter 1; MS salts, B5 vitamins, 1.1 µM BA, 0.3 µM GA₃, 3% sucrose (w/v) and 2.2g/L Phytagel (Sigma-Aldrich, Oakville, Canada). After a minimum
of four weeks in culture, explants with 3-4 internodes were excised and used for experiments. The explants were transferred to liquid media systems, alternative gelling agent medium, medium supplemented with activated charcoal, pH buffers, or AIP. All media were adjusted to a pH of 5.7 – 5.8 before adding the gelling agent (when present) and autoclaved for 20 minutes at 121°C and 21kpa. Once transferred into the media, plants were maintained in growth chamber at 16 hour photoperiod (40µmolm⁻²s⁻¹) provided by cool fluorescent lamps (Osram Sylvania Lts., Mississauga, Ontario), at 24±2°C.

3.2.2 Liquid State Media:

Plants that were transferred into liquid media were divided between test tubes or jars with support systems as shown in Figure 3.4. Individual explants were cultured in test tubes containing 12 mL of liquid media supplemented BA (0.0, 0.5, 1.1 or 2.2 µM). The media lacked a gelling agent with the exception of a control that contained 2.2 g/L Phytagel. A total of 30 explants were cultured in liquid or semisolid media in test tubes with three replications per treatment. Observations for shoot response were taken after four weeks of culture.

Glass jar culture systems were prepared with four support mechanisms: small beads, marbles, rocks and no support system as the control. There were replications of four jars for each treatment with approximately 20 mL of culture medium in each. Each jar received two explants with 3-4 internodes maintained upright by the support system. After four-weeks of culture, plants were observed for overall survival and health.
3.2.3 Gelling Agent:

Plants were subcultured into Magenta GA7 culture vessels (PhytoTechnology, Kansas, USA) containing 50 mL of culture media solidified with three levels of agar (Agar A111, PhytoTechnology, Kansas, USA) (0.2, 0.4, 0.6%) or Phytagal (2.2, 2.6, 2.8%). A total of 10 plants for each treatment were cultured with two plants per box for a total of five replicates per treatment. All cultures were maintained in growth chambers with a 16 hour photoperiod. Observations for plant growth and liquification of media were recorded after six weeks of culture.

3.2.4 Activated Charcoal:

Nodal segments were subcultured as described previously into medium supplemented with AC (0.0, 0.4, 0.6 or 0.8% w/v) and maintained in standard growth conditions for eight weeks. Four explants per Magenta GA7 culture vessels (PhytoTechnology, Kansas, USA), with four boxes for each treatment were maintained in a growth chamber. Observations were recorded including the degree of liquification of media, number of shoots, number of internodes, and shoot height.

3.2.5 pH Buffer:

To maintain the pH of culture medium, 0.2 M sodium phosphate dibasic heptahydrate was added into the culture medium, to maintain pH between 5.6-5.8. The pH buffer was also tested in combination with activated charcoal (AC) (0.6% w/v) with a total of four treatments; a control without AC or pH buffer, pH buffer alone, AC alone and AC with pH buffer. Approximately 50 mL of media with 2.2g/L Phytagal (Sigma-Aldrich, Oakville, Canada) was sterilized in each Magenta GA7 culture vessels. Four
explants were cultured per vessel with four boxes per treatment, and grown in culture conditions as described previously. Plants were maintained for approximately seven weeks until severe liquification of the control was observed and liquification, survival, appearance, health of the plants and any abnormalities were recorded.

3.2.6 AIP Treatment:

Magenta GA7 culture vessels (PhytoTechnology, Kansas, USA) were prepared containing approximately 50mL of culture media with AC at 0.6% (w/v) and AIP (0, 1, 2 or 5 µM). Four boxes of each treatment containing four explants each were transferred to a growth chamber. After seven weeks, media was examined for liquification and plants were observed for overall health, height, and number of shoots.

3.2.7 AIP Leaf Sample Preparation for Antioxidant and Phenolic Assay:

Seven week old plants cultured on each of the four AIP treatments were harvested for leaves to conduct a DPPH (2,2-diphenyl-1-picrylhydrazyl) antioxidant scavenging assay and the Folin – Ciocalteu total phenolic assay, to determine the effect of the inhibitor on phenolic and antioxidant levels in the plant. Holy basil plant leaves were harvested from each culture vessel and the fresh weights were measured. Harvested leaf tissue from each treatment was added to a 15 mL centrifuge tube (Fisher Scientific, Canada) and flash frozen using liquid nitrogen. Leaf tissue in 15 mL centrifuge tubes was dried using a lyopholizer (Freezone 4.5; Labconco, Kansas City, USA) for a minimum of 48 hours and dry weights were measured. Dried leaves were then ground in the 15 mL centrifuge tube (Fisher Scientific, Canada) and 75% acetone or 80% methanol at a ratio of 100 µL per 10 mg of tissue was added. The extraction
process was completed in a sonicating water bath (Branson 3510, Danbury, USA) for three hours. Tubes were centrifuged for 10 minutes at 1500 RPM and the supernatant was diluted into 1:10, 1:50 and 1:100 ratios.

3.2.7.1 Folin – Ciocalteu Total Phenolic Assay:

Leaf tissues were extracted in methanol to perform a Folin–Ciocalteu reagent (FCR) phenolics assay (Katsube et al., 2004). Phenolic quantities were estimated by comparing to a gallic acid standard (Sigma-Aldrich, Oakville, Canada) and determining the gallic acid equivalencies (GAE). Samples, blanks and standards were added to a clear flat bottom 96 well plate (Corning, USA) at a volume of 10 µL, with four replicates of standards, 75% acetone blank and water blank with three replicates of samples in addition to a sample blank. All wells except for blanks had 100 µL of 1:10 FCR (MP Biomedicals, USA) added. After five minutes, 80µL of 0.25 M Na$_2$CO$_3$ was added to each of these wells. To all blank wells, 180 µL of distilled water was added. After a dark incubation period of 60 min, absorbance was measured using Synergy H1 microplate reader (Biotek, Winooski, USA) at 715nm. Total phenolics in the extracts were estimated as compared to the standard curve generated with gallic acid standards ranging from 62.5-1000 mg/L (Figure 3.1). These values were used to estimate the amount of phenolics in dry plant tissues. Samples at the 1:50 dilution fell within the linear range and were used for analysis.

3.2.7.2 DPPH Assay:

Leaf tissue extracted in 75% acetone was used to determine antioxidant potentials. Samples were compared to trolox and expressed as trolox equivalency.
(µmolTE/g dry tissue) (Sigma-Aldrich, Oakville, Canada). Samples, blanks and standards were added to a clear flat bottom 96 well plate (Corning, Corning, USA) at a volume of 25 µL/well with four replicates of standards, 80% methanol and 75% acetone blanks, and three replicates of sample with an additional sample blank each. 200 µL of 150 µM DPPH solution (Sigma-Aldrich, Oakville, Canada) was added to all wells excluding blanks, which received 200 µL of 80% methanol. Plates were then incubated in the dark for six hours and read using Synergy H1 microplate reader (Biotek, Winooski, USA) at 517nm.

Samples were corrected with sample blanks and were compared to the Trolox standard curve to determine the antioxidant potential of the samples in relation to their dry weight, volume and dilution factor (Figure 3.2). Samples at the 1:50 dilution fell within the linear range and were used for analysis.

3.2.8 Trichome Analysis:

Plants grown in the presence of AIP (0-5 µM) were imaged using a scanning electron microscope to analyze trichome development. Leaf tissues were removed and photographed using an S-570 SEM (Tokyo, Japan) with Quartz PCI software (Quartz Imaging Corp., Vancouver, Canada).

Images were analyzed using ImageJ 1.47v (National Institute of Health, USA) to determine trichome number, glandular trichome diameter and area. For the number of trichomes, three 500 x 500 µm areas were randomly selected and the number of hairy, small glandular and large glandular trichomes was counted. The diameter of three randomly selected large and small trichomes were measured using the longest distance
across the structure and averaged for each treatment and tissue type. Comparisons of
the number, diameter, and area comparing all three types of trichomes between the
adaxial, abaxial leaf side and petiole of each AIP treatment were done.

3.2.9 Statistics:

All experiments were conducted using a completely randomized design and
analysis of variance was conducted using JMP version 11 for Windows (SAS Institute
Inc. Cary, NC). Means were compared using Tukey’s test (P< 0.05). The data are
presented as means ± standard error of means.
Figure 3.1 Corrected absorbance of gallic acid standards (mg/L) for the Folin-Ciocalteu assay to determine phenolic content of leaf tissue.
Figure 3.2: Standard Curves of the standard trolox for each of the DPPH assays to determine antioxidant activity in the leaf tissue.
3.3 Results:

3.3.1 Liquid and Solid Media:

Shoot growth was not significantly different between liquid and solid medium (Figure 3.3). However, while there were no significant differences in plants cultured in liquid media they generally started to decline before the four week subculturing period. Plants in liquid media exhibited hyperhydric symptoms and appeared to have decreased leaf growth and a reduced ability to support themselves.

Nodal segments cultured in glass jars (Figures 3.4) initially grew well. However, shoots started to show signs of necrosis and browning of leaves after four weeks of growth and often failed to survive. Shoots appeared less hyperhydric compared to those growing in culture tubes with liquid culture medium. Shoots were not submerged, but plants were unable to survive long-term in these conditions and did not multiply in this system.

3.3.2 Gelling Agents:

The media with agar at all levels was able to reduce the frequency of subculturing. However, shoots cultured on agar at 0.8% and above were significantly shorter than those on Phytagel (Figure 3.5) and at all concentrations agar based media resulted in fewer shoots than Phytagel (Figure 3.6). There were no significant differences in shoot height or shoot number among the concentrations of Phytagel.
3.3.3 pH Buffer (0.2M sodium phosphate dibasic heptahydrate)

The pH of the liquefying medium changed from the original pH of 5.7-5.8 to 3.9 to 4.1 after four weeks of culture. The destabilization of the media and need to subculture was severe after seven weeks and was prevalent in medium without buffer. The plants displayed leaf browning and necrosis, and some had become completely submerged in liquefied media. In contrast, medium supplemented with the pH buffer maintained its integrity (Figures 3.7, 3.8). Although the medium maintained its structure the overall survival of the plants was reduced (Figure 3.9). Medium with pH buffer resulted in 100% death in all replications while medium without pH buffer had a 25% survival rate.

The use of pH buffer in combination with AC improved the survival rate of plants to 32%, whereas the survival rate was 50% in medium containing AC and no pH buffer. Plants grown with 0.2M pH buffer and activated charcoal displayed deformed growth behaviour (Figures 3.8A, B). The leaves were thinner, lighter in colour, and more plentiful. The plants were lacking branches and remained stunted compared to the AC treatment alone.

3.3.4 Activated Charcoal:

Overall, the visual health of plants cultured with activated charcoal was improved and the frequency of subculture also decreased due to media remaining semi-solid. Plants cultured with 0.6% AC had improved growth characteristics compared to the control (Figures 3.7B, C; 3.8C, D), including an increased number of shoots per explants (Figure 3.10) with an average of 2.5 (SE ± 0.26) in the control and 3.9 (SE ± 0.19) with AC.
Figure 3.3 Percentage of holy basil shoots responding in solid and liquid media with various level of BA (0.0, 0.5, 1.1, 2.2 \( \mu \text{M} \)). Columns with different letters are significantly different (\( P<0.05 \))

Figure 3.4 Holy basil shoots growing in liquid medium with support of (A) marbles (B) rocks (D) small beads and (C) without any support, photographs shows plant growth response after 4 week of initial culture
Figure 3.5 Average shoot height of nodal segments transferred onto culture medium containing Phytagel (P) or agar (A) at different concentrations after six weeks. Columns with different letters are significantly different (P<0.05).

Figure 3.6 Average number of shoots on nodal segments transferred onto culture medium containing either Phytagel (P) or agar (A) at different concentrations after six weeks. Columns with different letters are significantly different (P<0.05).
Figure 3.7 Holy basil shoots growing in culture medium with pH buffer (A) and without pH buffer (B and C) for 7 weeks

Figure 3.8 Holy basil shoots growth on media containing activated charcoal (0.6%) with pH buffer (A and B) and without pH buffer (C and D) for 7 weeks
Figure 3.9 Average percentage of dead plants after 7 weeks in culture: culture medium with (1) pH buffer at 0.2M (2) no pH buffer or AC (3) 0.2M pH buffer and 0.6% AC (4) no pH buffer and with 0.6% AC. Columns with same letter indicate no significant difference (P<0.05)
There were no significant differences between the number of internodes with or without AC (Figure 3.11). The average shoot length of plants was measured after four weeks in culture, and 0.6% (w/v) was significantly longer than the control at 57.4mm (SE ± 1.21) (Figure 3.12).

3.3.5 AIP Treatment:

The use of AIP at 2 μM resulted in significantly higher number of shoots per explant (6.33 SE ± 0.68) compared to all other treatments (Figure 3.14). Plants grown on AIP were not significantly different in height than the control plants (Figure 3.14). As well, media containing AIP in combination with AC maintained its semi-solid state (Figure 3.13).

3.3.6 Phenolic and DPPH Assay of AIP Treatment:

Plants cultured in AIP at 1 and 5 μM were not significantly different in phenolic content from the control (Figure 3.15). However, a significant increase was observed with leaf tissue of plants grown in 2 μM AIP (57.48 GAEmg/g dry tissue). All AIP treatments had a significant increase in antioxidant potential when compared to the control (Figure 3.16).
Figure 3.10 Average number of shoots recorded when explants cultured on media containing activated charcoal (0 – 0.8% w/v) after four weeks of culture. Columns with same letter indicate no significant difference (P<0.05)

Figure 3.11 Average numbers of internodes recorded when explants cultured on media containing activated charcoal (0 – 0.8% w/v) after four weeks of culture. Columns with same letter indicate no significant difference (P<0.05)
Figure 3.12 Average shoot length recorded when explants cultured on media containing activated charcoal (0 – 0.8% w/v) after four weeks of culture. Columns with same letter indicate no significant difference (P<0.05).

Figure 3.13 Holy basil explants grown in culture medium containing 0.6% AC with four levels of AIP for 7 weeks. A) 0 µM B) 1 µM C) 2 µM D) 5 µM
Figure 3.14 Height (cm) and number of holy basil shoots cultured in AIP (0-5 µM) for 7 weeks. Columns with same letter indicate no significant difference (P<0.05)
3.3.7 Trichome Development:

Plants grown in 0, 1 and 2 μM AIP had morphologically similar trichomes (Figure 3.17A, B, C), leaf epidermis and stomata. In the epidermis of the 5 μM AIP treatments, the cells were more pronounced and jagged compared to the control (Figure 3.17D). For the small glandular trichomes (capitate), the majority were deformed without being circular or oval as seen in the other three treatments.

Plants examined using florescence microscopy demonstrated that the trichomes autofluoresce yellow, potentially indicating the presence of phenolic compounds (Figure 3.18). The number of trichomes exhibiting this trait was similar among AIP treatments. The average number of trichomes found on the leaf surface of the four AIP treatments varied between trichome types. For peltate, capitate or hairy trichomes, there were no significant differences (Figure 3.19). The area of peltate trichomes at low AIP concentrations (1 and 2 μM) was significantly greater (Figure 3.20), with no differences in diameter. Plants grown in AIP at 5 μM had a significantly smaller diameter for peltate trichomes than the control (Figure 3.21). Capitate trichomes displayed no significant differences in area or diameter among any of the treatments (Figure 3.22, 3.23).
Figure 3.15 Phenolic content of holy basil leaves grown *in vitro* in different AIP concentrations after 7 weeks. Columns with same letter indicate no significant difference (P<0.05).

![Phenolic Content Graph](image)

Figure 3.16 Antioxidant potential of holy basil leaves grown in AIP conditions after 7 weeks. Columns with same letter indicate no significant difference (P<0.05).

![Antioxidant Activity Graph](image)
Figure 3.17 SEM images of holy basil leaf samples grown with different concentrations of AIP (A) 0 µM (B) 1 µM (C) 2 µM and (D) 5 µM. All scale bars are 200 µm.

Figure 3.18 Photographs of the adaxial side of leaf tissue grown in vitro with four AIP treatments after 8 weeks in culture using fluorescence microscopy (10x). AIP concentrations: (A) 0.0 µM (B) 1.0 µM (C) 2.0 µM and (D) 5.0 µM.
Figure 3.19 Average number of three different trichome types (peltate, capitate, hairy) per 500 x 500 µm² area of plants grown in AIP concentrations of 0, 1, 2 and 5 µM as analyzed through scanning electron micrograph images. Columns with same letter indicate no significant difference (P<0.05)
**Figure 3.20** Average area (µm$^2$) of peltate trichomes of leaves grown in AIP concentrations of 0, 1, 2 and 5 µM analyzed from scanning electron micrograph images. Columns with same letter indicate no significant difference (P<0.05)

**Figure 3.21** Average diameter (µm) of peltate trichomes of leaves grown in AIP concentrations of 0, 1, 2 and 5 µM analyzed from scanning electron micrograph images. Columns with same letter indicate no significant difference (P<0.05)
Figure 3.22 Average area (µm²) of capitate trichomes of leaves grown in AIP concentrations of 0, 1, 2 and 5 µM analyzed from scanning electron micrograph images. Columns with same letter indicate no significant difference (P<0.05)

Figure 3.23 Average diameter (µm) of capitate trichomes of leaves grown in AIP concentrations of 0, 1, 2 and 5 µM analyzed from scanning electron micrograph images. Columns with same letter indicate no significant difference (P<0.05)
3.4 Discussion:

3.4.1 Liquid and Solid Media

Liquid culture systems are beneficial as they can increase the production of secondary metabolites in medicinal plants as well as improve multiplication (Paek et al., 2005). In the case of holy basil, liquid culture systems resulted in reduced proliferation and multiplication. Plants exhibited hyperhydricity, leading to necrosis of the leaves, and weakened growth. Plants were unable to survive long term in these systems, suggesting that liquid culture systems are not suitable for the micropropagation of holy basil using the culture conditions described in this study. Similar results were observed in *Scutellaria* species, a medicinally important genus in the same family (Lamiaceae), where agitated and non-agitated liquid culture systems reduced multiplication rates and induced a hyperhydric responses (Tascan and Adelberg, 2010). This is also common in woody plant species such as apple (Paek et al., 2005) and Eucalyptus (Whitehouse et al., 2002) where hyperhydric plants were produced in a temporary immersion bioreactor liquid system.

However, root production in holy basil was higher in flasks supplemented with liquid media compared to solid media (Shilpa et al., 2009), though no observations on the effects on shoot production were mentioned.

With the use of liquid medium for multiplication of holy basil, it has been determined that these methods for propagation are a detriment to the growth and development of plants.
3.4.2 Gelling Agents

Agar and Phytagel (Sigma-Aldrich, Oakville, Canada) are commonly used gelling agents in plant tissue culture, but they differ in their origin and effects (George et al., 2008). This difference was evident in the current study with holy basil multiplication rates and need for subculturing. The multiplication rate of holy basil was significantly lower with the use of agar than on Phytagel. In several previous studies on *O. sanctum*, *in vitro* cultures were maintained on 0.7-0.8% agar for regeneration from various explant sources including shoot (Pattnaik and Chand, 1996), inflorescence (Singh and Sehgal, 1999), callus (Hakkim et al., 2007; Xiong Lim et al., 2009), root culture (Shilpa et al., 2009) and axillary buds (Gogoi and Kumaria, 2011). These studies demonstrated successful regeneration of holy basil through agar based media without liquification of culture medium. However, based on the results of the current study, these protocols would likely be improved with the replacement of agar with Phytagel.

The relatively negative effects of agar on *in vitro* cultures have been observed in a variety of plant species. Debergh (1983) and Hakkaart and Versluijs (1983) both found that the use of 1.1-1.2% agar decreased shoot proliferation and growth when compared to lower concentrations of agar. Gellan gum was superior in supporting regeneration of sugar cane cultures (Anders et al., 1988), callus regeneration and shoot development in rice (Joyia and Khan, 2013), adventitious bud growth in pear (Chevreau et al., 1997) and shoot length, weight and number in banana (Kaçar et al., 2010). However, improved shoot regeneration and growth observed with the use of Phytagel in holy basil was also accompanied by destabilization of the medium and necessity for frequent subculturing. The medium solidified with agar maintained its integrity throughout the
culture period. Differences in the effects of Phytagel and agar may result from different chemical compositions of their sources. Phytagel is a bacterial derived gelling agent and agar is derived from algal species (PhytoTechnology laboratory information sheet, 2011). While the underlying mechanism is unknown, it appears that holy basil produces exudates that interfere with the exopolysaccharide matrix of Phytagel, but have little effect on the stability of the agar matrix.

3.4.3 pH buffer:

Ideal pH for plant growth ranges between 5.5-6.0 to optimize ion availability to the plants (George et al., 2008). The pH can be affected during the autoclaving process by hydrolysis of organic compounds, or while in culture through uptake and release of compounds by the plant such as N03− and NH4+ (Skirvin et al., 1986). The pH of culture media can affect plant growth and the efficiency of gelling agents, and could influence the health of the plants in vitro.

The use of sodium phosphate dibasic heptahydrate as a pH buffer had a negative impact on the growth of the plants, causing death and necrosis of the majority of them. Although the use of buffer was deemed not useful for a propagation system due to its toxicity, the media remained stable. Similarly, Parfitt et al. (1988) observed a negative impact of pH buffer MES (2-(N-morpholino) ethanesulphonic acid) and TRIS (Tris (hydroxymethyl) aminomethane) for shoot tip and callus regeneration in different plant species. Apple cultures showed reduced adventitious root initiation with low levels of MES (Klerk et al., 2008).
3.4.4 Activated charcoal

Activated charcoal (AC) is a common additive used in plant tissue culture primarily for its adsorbent properties (Thomas, 2008). These properties have led to improvements in shoot multiplication, elongation and rooting along with many other positive effects for *in vitro* growth (Thomas, 2008). However, activated charcoal can also absorb plant growth regulators and thereby alter their efficacy (George et al., 2008).

In the present study the use of 0.6% (w/v) activated charcoal improved the multiplication from 2.5 to 4 shoots per explant. Additionally, a noticeable improvement in browning of leaves, increased survival rates, and better vegetative growth were observed. As well, the AC maintained the integrity of the semi-solid matrix for up to eight weeks. While the use of AC in holy basil cultures has not previously been reported, it improves shoot production and overall health in a variety of other culture systems. Orchids demonstrated improved shoot and root growth development in the medium supplemented with 0.2% AC (Moraes et al., 2003). Improved shoot multiplication and elongation was found in date palm with the addition of AC compared to basal medium (Abdulwahed, 2013). Improvements in elongation in cashew was found with the addition of activated charcoal (Boggetti et al., 1999). In the medicinal plant family Piperaceae (Madhusudhanan and Rahiman, 2000) and yew trees (Chang et al., 2001), AC decreased browning.

The use of activated charcoal in micropropagation of holy basil has not been previously reported. The natural properties of AC in maintaining pH and adsorbing
inhibitory compounds may be the cause of this positive response. The elimination of browning, stimulation of shoot proliferation and decreased liquification of media indicate that the use of 0.6% activated charcoal in semi-solid culture medium is ideal for large scale production of holy basil.

3.4.5 AIP Treatment

Increased multiplication, low browning and minimal need of subculturing are necessary to improve the efficiency of holy basil micropropagation. Plants grown on activated charcoal (0.6%) showed significant improvements in overall health of plants and multiplication, while extending the length of time between subculturing. The addition of AIP in culture medium with activated charcoal was examined to further determine its effects on in vitro cultures.

AIP is a competitive enzyme inhibitor that blocks the first step in the phenylpropanoid pathway, thus decreasing the downstream products such as phenolic acids and flavonoids (Appert et al., 2003; Zoń and Amrhein, 1992). Oxidative browning of plants is generally thought to be related to this pathway. Phenolic compounds are subsequently produced from the wounding response of subculturing (Jones and Saxena, 2013). However, these phenolic compounds along with holy basil's complex chemical composition are key to the medicinal qualities of the species (Kumar et al., 2013). Because of this, not only was the visible physical response viewed with holy basil but comparison of phenolic content and antioxidant potential was conducted to ensure the plant remained medicinally active for analysis and screening of elite plants.
The use of 2 µM AIP increased the average number of axillary buds (6.3) compared to the control while decreasing the amount of browning observed in leaf tissue. Use of 1 µM and 5 µM also improved these parameters, but not significantly. However, there were no significant differences in the phenolic content or antioxidant potential among any of the AIP treatments. This indicated that although there may be effects on the phenolic production, it doesn’t significantly impair the production of beneficial medicinal products, likely due to the low levels of AIP used.

The use of AIP has not been previously described for use in holy basil but AIP has been used to decrease browning and phenolic content in other plant species. For example, AIP inhibited the browning of lettuce after cutting and cold storage (Peiser and López-Gálvez, 1998; Hisaminato et al., 2001). However, this was observed at higher concentrations of AIP (50 µM and 10 µM, respectively). Jones and Saxena (2013) also observed reduced browning in *Artemisia annua* at 10 µM. The authors found that the browning in American elm and sugar maple callus was also reduced with 1mM concentrations of AIP. Using duckweed fronds, Janas et al. (1998) observed a negative multiplication rate with the increase in AIP concentration and produced morphologically different fronds, believed to be due to accumulation of starch. This negative response could have been associated with the significantly higher levels of AIP used than that used in this research. In birch, the use of AIP treatments decreased growth (Keski-Saari, 2005), but, AIP improved growth of *Salix pentandra* (Ruuhola and Julkunen-Titto, 2003). In holy basil, an improvement in growth and development with the addition of AIP through an increase in multiplication, decrease in browning and elimination of the liquification process in long-term storage was observed.
The use of AIP in other plant systems has resulted in decreased phenolic production. In *A. annua*, the use of 100 µM significantly decreased phenolic content in cotyledon callus (Jones and Saxena, 2013). This was also demonstrated in birch with an inverse relationship, where an increase in AIP (5-30 µM) decreased the concentration of total phenolics (Keski-Saari, 2005). In the case of holy basil, there was a significant positive difference in phenolic content of plants cultured on 0-5 µM AIP. This could have been associated with the lower levels of AIP used.

Trichome development was impacted by the inclusion of AIP. Significant differences were observed between treatments and the control on quantitative data. Visual alterations were apparent in the concentrations of AIP at 5 µM including deformed epidermis, stomata and trichome development. In duckweed, the application of AIP at higher concentrations (10 µM) resulted in morphological changes in leaf tissues, mainly the aerenchyma and mesophyll. This was hypothesized to be due to AIP’s indirect impact on increasing auxin levels in the plant (Gitz et al., 2004).

3.5 Conclusion

The use of pH buffer, different gelling agents and liquid state media did not improve the growth or survival rates of *in vitro* culture of holy basil. However, the use of 2 µM AIP and AC at 0.6% (w/v) produced higher numbers of shoots per explant, increased shoot height, and increased the efficiency of the micropropagation process. This was additionally beneficial as it did not compromise the medicinal value of the plant. Both phenolics and antioxidant potential remained consistent or greater when compared to the control, allowing for further screening of *in vitro* grown plants to not be
compromised. These advances have facilitated an efficient method for multiplication of holy basil through a semi-solid culture system that can be used for mass production of genetically uniform plants.
4.0 Chapter 4: Identification of Elite Accessions of Holy Basil (Ocimum sanctum L.) with high Medicinal Value

Abstract:

Holy basil plants from five sources of seed from Canada and India were established \textit{in vitro} to identify elite plants with high antioxidant activity. An accession derived from an Indian seed lot was found to have the highest antioxidant capacity at 1452.08 µmolTE/g and was subsequently named “Vrinda”. The antioxidant potential and phenolic content of plants derived from seed produced by selfing “Vrinda” were measured under different growth conditions including greenhouse, field and \textit{in vitro} conditions. “Vrinda” derived plants were also analyzed for the concentration of three neurotransmitters, melatonin, serotonin and GABA. Based on its high antioxidant potential and high neuro-chemical content, “Vrinda” was identified as an elite accession of holy basil with potentially high medicinal value that can be propagated via \textit{in vitro} propagation techniques.

4.1 Introduction:

Holy basil (Ocimum sanctum L.) is a plant known for its high medicinal value and religious significance in ancient Indian texts. Ayurveda is one of the ancient systems of traditional medicine practiced in India (Gurib-Fakim, 2006). The Veda, or text, details many aspects of whole body healing through plant derived medicines. These texts describe the uses of holy basil (Tulsi), also known as the “Queen of Herbs” and “Elixir of Life” (Engels and Brinckmann, 2013). A number of beneficial properties of Holy basil have been described in the literature including adaptogenic, antioxidant, antimicrobial,
antiviral, antifungal, antiseptic, antipyretic, spasmodic, expectorant, nerve and as a stimulant (Engels and Brinckmann, 2013; Das and Vasudevan, 2006; Gupta et al., 2007; Wangcharoen and Morasuk, 2007). It also helps in a wide range of health problems including: abdominal issues, digestive problems, throat and mouth infections, headache, fever, microbial ailments, tumours, skin and eye issues, aging, mental capacity to name a few (Mondal et al., 2009). The world health organization monographs further describe holy basil’s benefits for the prevention of many ailments including arthritis, asthma, bronchitis, cold, diabetes, fever, influenza, ulcer and rheumatism (World Health Organization, 2002). In Canada, leaves and seeds of holy basil are classified under the natural health products for active ingredients (Singh et al., 2012; Kumar et al., 2013; Engels and Brinckmann, 2013; Government of Canada, 2004e).

Consumption of plant secondary metabolites by humans can assist them against acute and chronic illnesses (Crozier et al., 2006). Beneficial effects of plants have been documented for thousands of years and medicinal plants have been incorporated into many traditional medicine systems including Unani, Chinese traditional medicine, Ayurvedic and Native American texts (Gurib-Fakim, 2006). The knowledge in use today with 90% of the world’s population using plant derived medicine and 70% of Canadians use herbal medicines (Agri-Food Canada, 2012). Over 13 000 plants are described in traditional medicinal systems, with the USA using over 1 800 plants for medicinal purposes (Agri-Food Canada, 2012). A number of pharmaceuticals such as morphine and aspirin were originally derived from plants (Agri-Food Canada, 2012). However,
traditional medicines do not focus on a single compound, but use whole plants and/or extracts that contain a plethora of secondary metabolites found in plants.

High levels of secondary metabolites such as phenolics and terpenes in holy basil are thought to be responsible for its medicinal value (Wangcharoen and Morasuk, 2007). Holy basil's major constituents include compounds such as eugenol, urosolic acid, methyl-eugenol, linalool, caryophyllene, methyl carvacol (Kumar et al., 2013). Major quantities of phenolics are found in the leaf tissue and the tissue can have up to 90% methyl-eugenol and eugenol. Other phenolics such as cirsimelineol, circimatin, isothymusin, apigenin, rosmarinic acid and flavonoids orientin and vicenin are also present in high quantities in the leaf (Singh et al., 2012). Since there is no single compound responsible for the medicinal effects of holy basil, elite accessions cannot be identified based upon a single marker compound. However, because the medicinal effect is thought to be related to the phenolic content of plants, the total phenols could serve as a preliminary screening method. Another approach that has been used in a variety of medicinal plants is based upon their antioxidant potential (Atawodi, 2005; Pietta et al., 1998; Lee et al., 2003). These approaches may provide a method to first screen and identify accessions for further study and selection.

Recently, many medicinal plants have been found to contain human brain chemicals such as melatonin (N-acetyl-5-methoxytryptamine), serotonin (5-hydroxytryptamine) and gamma-aminobutyric acid (GABA). Melatonin was originally thought to be only found in vertebrate animals, but was recently discovered in bacteria, fungi and plants (Reiter and Tan, 2001). In humans, melatonin regulates circadian rhythms and acts as a strong antioxidant against reactive oxygen species such as hydroxyl radicals, hydrogen
peroxide, nitric oxide, peroxynitrite anion, peroxynitrous acid and hypochlorous acid (Reiter and Tan, 2001; Tan et al., 2012). In plants, melatonin has many beneficial effects, it help plants against oxidative stresses, and regulate circadian rhythms (Cole et al., 2007; Reiter and Tan, 2001; Tan et al., 2012). Serotonin, the precursor to melatonin, is a well-known neurotransmitter in humans, and has been associated with a range of health benefits (Mohammad-Zadeh et al., 2008). Additionally, GABA has been shown to improve mood disorders, seizures and epilepsy in humans (Houser, 1991; De Deyn et al., 1990; Petty, 1995). In plants GABA also acts as a signalling molecule, involved with adapting to stress and related metabolism (Kinnersley and Turano, 2000; Fait et al., 2008).

In this study, there were three main objectives: 1) to evaluate the variation in total phenol content and antioxidant potential in a seedling population to identify elite accessions, 2) to determine the effect of different growth conditions on antioxidant potential and 3) to determine the melatonin, serotonin and GABA content of the elite plants.

4.2 Materials and Methods:

4.2.1 Plant Source Material

Seeds were obtained from five different sources; “Richter” (Richter’s Seeds; Goodwood, ON), “Horizon” (“Horizon” Herbs, LLC, Williams, OR, 97544, USA) and other three of seed lots, “Tulsi”, “India”, and “Garden” obtained from suppliers in Meerut City, UP, India. Seeds were sterilized by agitation in 10% (v/v) bleach solution (Clorox, 5.5%) for four minutes, followed by three rinses in sterilized deionized water at three
minutes each rinse. Seeds were then transferred to Petri plates containing media comprised of MS salts (Murashige and Skoog, 1962) with 2% sucrose, B5 vitamins (Gamborg et al., 1968), and solidified with 2.2g/L Phytagel (Sigma-Aldrich, Oakville, Canada). For seed germination, the plates were kept at 24 ± 2°C under a 16 hour photoperiod (40µmolm⁻²s⁻¹) provided by cool fluorescent lamps (Osram Sylvania Lts., Mississauga, Ontario). Once seedlings passed the cotyledon stage, they were transferred to Magenta GA7 culture vessels (PhytoTechnology, Kansas, USA) containing the same media as described above. Sub-culturing was done using culture medium previously optimized for holy basil growth (Chapter 2). The shoots were multiplied from each heterozygous germinated seedling and maintained separately by subculturing until enough leaf material was acquired to perform the subsequent assays.

4.2.2 Sample Preparation for Antioxidant and Phenolic Assay:

Approximately 0.05g of dry leaf tissue was used for the DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay. Harvested leaves were added to a 15 mL centrifuge tube (Fisher Scientific, Canada) and flash froze in liquid nitrogen. The tissues were lyophilized (Freezone 4.5; Labconco, Kansas City, USA) for 48 hours. Dried leaves were ground using a vortexer and ball bearings in the 15 mL centrifuge tube (Fisher Scientific, Canada), and 75% acetone for DPPH or 80% methanol for Folin- Ciocalteu total phenolic assay (FC) was added at a ratio of 100µL:10mg of tissue. The extraction process was completed by keeping the centrifuge tubes in a sonicating water bath (Branson 3510, Danbury, USA) for three hours. The tubes were then centrifuged for 10 minutes at 1500 rpm and the supernatant was transferred to new tubes and diluted to 1:50 and 1:100 ratios with the extraction solvent.
4.2.2.1: DPPH Antioxidant Assay:

Holy basil extracts samples, alcohol blanks and trolox equivalency standards (Sigma-Aldrich, Oakville, Canada) were added to clear flat bottom 96 well microplates (Corning, Corning, USA) at a volume of 25 µL per well. For each standard and blank, four replicates were used. Samples were replicated three times with an additional standard blank for each extract. 200 µL of 150 µM DPPH solution (Sigma-Aldrich, Oakville, Canada) was added to all wells excluding alcohol and sample blanks, which received 200µL of 80% methanol. Plates were then incubated in the dark for six hours and the absorbances were measured with a Synergy H1 microplate reader (Biotek, Winooski, USA) at 517nm.

Samples were corrected by the sample blank and compared to the Trolox standard curve (31.25 – 500 µmol/L) to determine of the antioxidant capacity of each sample as trolox equivalency (TE).

4.2.2.2 Folin- Ciocalteu Phenolic Assay:

For the gallic acid standard, 80% methanol and water blanks, 10 µL aliquots were pipetted into clear flat bottom 96 well microplates (Corning, Corning, USA) with four replicates each. Samples were pipetted into the same plate at a volume of 10 µL, replicated three times with an additional sample blank for each. To all standard and sample wells, 100 µL of FC reagent (MP Biomedicals, USA) was added. After five minutes, the same wells received 80 µL of 0.25 M Na₂CO₃. To all methanol, water and sample blank wells, 180 µL of distilled water was added. After a dark incubation period
for an hour, microplates were read using Synergy H1 microplate reader (Biotek, Winooski, USA) at 715nm.

The absorbance values of tissue samples were compared to the gallic acid equivalent standard curve (62.5 – 1000 mg/L) to determine the gallic acid equivalency (GAE). Based on the dry weight, volume, and dilution factor, these values were used to estimate the phenolic content in the plant tissue. Samples at the 1:50 dilution fell within the linear range and were used for analysis.

4.2.3 Greenhouse Growth Conditions

Plants with high antioxidant capacity were transferred into the greenhouse. Roots of these plants were established using 0.5 µM IBA in combination with activated charcoal (0.6%). The plants were acclimatized to greenhouse condition by first putting them under mist beds. Conditions of the greenhouse were 24°C at 16 h light and 20°C for 8 h darkness. Plants were maintained in the greenhouse until maturity.

4.2.4 Quantification of melatonin, serotonin and GABA:

4.2.4.1 Sample preparation:

Three individual plants of each source, namely “Tulsi”, “Garden” and “India” were selected for determination of melatonin, serotonin and GABA. The leaf tissue from these plants was flash frozen using liquid nitrogen and dried in a lyophilizer (Freezone 4.5; Labconco, Kansas City, USA) for 48 hours. Samples were stored at -80°C until analysed at the University of British Columbia, BC.
Extracts were analyzed using previously described methods (Murch et al., 2010) with minor modifications. Samples were extracted in a darkened room with a single red light as the only light source using 100 µl of methanol: water: 0.1 N formic acid (80:19:1 v/v). Tissues were homogenized for 30 s (Kontes Pellet Pestle disposable tissue grinder, Fisher Scientific, Mississauga, ON) and centrifuged (16,000 x g, Eppendorf Microfuge) for one minute to settle particulate matter. The resulting supernatant was filtered (0.2 µm, Ultrafree-MC filtered centrifuge tubes; Millipore, MS, USA) prior to separation and quantification of MEL and 5HT. All samples were prepared in cool darkness in less than seven minutes to prevent light and temperature degradation of targeted compounds.

4.2.4.2 Chromatography and Mass Spectrometry

Melatonin, GABA and serotonin were separated from the extract by Ultra-Performance Liquid Chromatography (Acquity UPLC, Waters Corp, Mississauga, ON) on a reverse phase chromatography column (Waters BEH C18 UPLC column, 2.1 X 150 mm, 1.7 l µm; Waters, MA, USA). Compounds were eluted with a gradient of 1% aqueous formic acid : acetonitrile (0.0 - 4.0 min, 95:5 - 5:95 v/v, 4.0-4.5 min, 95:5—95:5 v/v, 4.5-5.0 min, 95:5 v/v) at 0.25 ml/min and 30 °C over five minutes with a two minute cleaning and re-equilibration period. Melatonin, GABA and serotonin were detected by time of flight mass spectrometry (ToF-MS; LCT Premier Micromass MS, Waters) using electrospray ionization (ESI) in the positive mode with m/z determination using the “V” configuration ion flight path and previously published standard voltages (Murch et al., 2010). Indoleamines were quantified by comparison to authenticated standards with purities greater than or equal to 98% (Sigma Aldrich, Oakville, Canada) for retention
time and mass accuracy. Averages of all plants from either greenhouse or field were combined to determine variation between growing conditions on the quantities of neurotransmitters.

4.2.5 Statistical Analysis:

All experiments were conducted using a completely randomized design. The analysis of variance was conducted using JMP version 11 for Windows (SAS Institute Inc. Cary, NC). Means were compared using Tukey’s test (P< 0.05). The data are presented as means ± standard error of means.

4.3 Results:

4.3.1 Screening of Holy Basil Plants for Antioxidant Capacity

Out of the five plants from Richter’s seed company screened using DPPH bioassay, plant named ‘R2’ was found to have the highest antioxidant potential with an average of 412.21 µmolTE/g. The range of five plants was between 120.77 - 412.21 µmolTE/g with an average of 206.26 µmolTE/g (Figure 4.1). The plants obtained from Richter’s Seeds demonstrated a narrow growth habit, small greenish-yellow leaves, and little or no branching when grown in vitro (Figure 4.2).

Twenty-seven individual plants of the seed lot from India named “Tulsi” were analyzed through the DPPH method. The average antioxidant potential of this seed lot was 389.37 µmolTE/g, but ranged from 69.37 – 1452.08 µmolTE/g (Figure 4.3). T5 was identified with highest antioxidant activity. These plants demonstrated quick growth in vitro with larger leaves and longer internodes (Figure 4.4).
Analysis of plants from the Indian seed lot “Garden” showed an average antioxidant potential of 160.47 µmolTE/g and ranged from 78.50 - 238.78 µmolTE/g (Figure 4.5). G5 had the highest antioxidant capacity. These plants were slow to grow and had little branching with small leaves (Figure 4.6).

Seventeen plants of seed lot “Horizon” were found to have an average antioxidant potential of 103.36 µmolTE/g ranging from 40.37 – 380.57 µmolTE/g (Figure 4.7). J2 had the highest antioxidant activity. Plants showed large leaves but had moderate to low levels of antioxidant potential (Figure 4.8).

Twenty-three plants of seed lot “India” had an average antioxidant potential of 80.54 µmolTE/g, ranging from 21.177 – 181.582 µmolTE/g (Figure 4.9) with I22 being significantly higher than most in antioxidant activity. These plants demonstrated good growth in vitro with large, light coloured leaves, and more branches. Leaves of this seed lot were curled and serrated rather than smooth and flat as seen in plants of other seed lots (Figure 4.10).
Figure 4.1 Antioxidant scavenging activity of plants from ‘Richter’ seed lot measured by DPPH antioxidant assay and compared to a trolox equivalent standard (µmolTE/g). Columns with same letter indicate no significant difference (P<0.05)

Figure 4.2 Four week old seedlings from “Richter” seed lot grown in vitro
Figure 4.3 Antioxidant scavenging activity of individual “Tulsi” plants grown in vitro when compared to a trolox equivalent (µmolTE/g) standard using DPPH antioxidant assay. Columns with same letter indicate no significant difference (P<0.05).

Figure 4.4 Photograph of seed lot “Tulsi” grown in in vitro conditions after four weeks in culture media.
Figure 4.5 Average antioxidant scavenging activity of plants from “Garden” seed lot compared to a trolox equivalent standard (µmolTE/g) using DPPH antioxidant assay. Columns with same letter indicate no significant difference (P<0.05).

Figure 4.6 Photograph of four week old seedlings of “Garden” seed lot grown in vitro conditions.
Figure 4.7 Average antioxidant scavenging activity of plants grown from “Horizon” seedlings in vitro when compared to a trolox equivalent (µmolTE/g) standard using DPPH antioxidant assay. Columns with same letter indicate no significant difference (P<0.05).

Figure 4.8 Photographs showing seed lot “Horizon” in culture after 4 weeks in vitro in culture media.
Figure 4.9 Average antioxidant scavenging activity of plants grown from “India” seedlings in vitro when compared to a trolox equivalent (µmolTE/g) standard using DPPH antioxidant assay. Columns with same letter indicate no significant difference (P<0.05)

Figure 4.10 Seed lot “India” growing in in vitro conditions at first subculture (A) and after four weeks in vitro (B) in culture media
4.3.2 The Plants with highest antioxidant capacity

The 20 plants with the highest antioxidant activity are shown in Figure 4.11. Out of these twenty plants, the five plants with highest antioxidant potential (1452.08 – 1261.55 µmolTE/g) were from the “Tulsi” seed lot. The plant with highest antioxidant potential (Figure 4.12), was named “Vrinda”. This not only showed high antioxidant capacity but also contained other desirable traits such as high \textit{in vitro} multiplication rates, large leaves and quick growth after subculturing.

4.3.3 Analysis of Greenhouse Grown “Vrinda” Plants

Mature “Vrinda” plants were multiplied and grown in the greenhouse to maturity. Leaf samples were taken from five individual “Vrinda” plants and analyzed for antioxidant and phenolic content. Phenolic content of greenhouse grown plants were between 66.6 and 93.4 GAEmg/g tissue (Figure 4.13). Antioxidant capacity of the plants ranged between 6741.77 – 7717.59 µmolTE/g (Figure 4.14).

Greenhouse grown “Vrinda” plants were compared to plants grown in field conditions (Figure 4.15). No significant difference was observed in terms of antioxidant activity between the field and greenhouse grown plants (Figure 4.16).
Figure 4.11 Top 20 plants of all varieties of average anti-oxidant scavenging activity compared to a Trolox equivalent (µmolTE/g) standard using DPPH antioxidant assay. Columns with same letter indicate no significant difference (P<0.05)

Figure 4.12 Photographs of four week old “Vrinda”, grown in *in vitro* conditions on multiplication culture medium.
4.3.4 Comparison between Greenhouse and *In Vitro* Grown “Vrinda” Plants

A higher phenolic content of 13.36 GAEmg/g was present in the roots of greenhouse grown “Vrinda” plants than other tissues and was significantly greater than leaf and stem of greenhouse and *in vitro* grown plants, and root samples (Figure 4.17). The other tissue samples ranged from 7.22 - 8.36 GAEmg/g in both greenhouse and *in vitro* samples.

4.3.5 Concentration of Neurotransmitters in Holy Basil:

Melatonin, serotonin and GABA levels were analyzed through UPLC to determine the concentrations in roots and leaves from greenhouse and field grown “Vrinda” plants. Levels of melatonin in leaves ranged from 327.66 - 364.31 ng/g of fresh weight in the greenhouse and 311.11 – 353.65 ng/g in the field grown plants (Figure 4.18). Levels of melatonin in the roots ranged from 303.82 – 451.85 ng/g in the greenhouse and 352.64 – 400.10 ng/g in the field (Figure 4.19). No significant difference in melatonin content was observed between roots or shoots grown in the greenhouse or field (Table 4.1).

Serotonin levels in the leaves and roots were much higher than melatonin levels in same organs. The serotonin level in the leaves ranged from 127.67 – 873.80 ng/g in the greenhouse grown plants and, 415.50 – 1205.00 ng/g in the field grown plants (Figure 4.20). The level of serotonin in the roots ranged from 153.25 – 815.54 ng/g in the greenhouse grown plants and 350.60 – 716.60 ng/g in field grown plants (Figure 4.21). No significant difference was observed between greenhouse and field for either roots or shoots (Table 4.1.).
Figure 4.13 Average phenolic content (GAEmg/g) from leaves of five different greenhouses grown “Vrinda” plants analyzed via the FC method. Columns with same letter indicate no significant difference (P<0.05)

Figure 4.14 Average antioxidant activity measured as a trolox equivalent (µmolTE/g) from leaves of five different greenhouse grown “Vrinda” plants analyzed via the DPPH method. Columns with same letter indicate no significant difference (P<0.05)
**Figure 4.15** Greenhouse grown (A) and outdoor grown (B) “Vrinda” plants showing the colour variation from green to purple in the leaves and stem
Concentrations of GABA were between 0.52 – 0.64 mg/g in the leaves of greenhouse grown plants and 0.54 – 0.71 mg/g in the leaves of field grown plants (Figure 4.22). GABA concentrations were detected in the roots ranging from 0.013 – 0.022 mg/g in greenhouse grown plants and 0.013 – 0.033 mg/g in field grown plants (Figure 4.23). No significant difference was observed in the GABA levels between roots and shoots in field and greenhouse grown plants (Table 4.1).

For *in vitro* grown plants, differences were observed in melatonin and serotonin levels, the plants from seed lot called “India” had significantly lower quantities of both melatonin and serotonin compared to the melatonin and serotonin content of the plants of “Tulsi” and “Garden” seed lots (Figures 4.24, 4.25).
Figure 4.16 Samples of greenhouse (GH) and field grown “Vrinda” tissue of stem, leaf and flower were analyzed for antioxidant activity comparing to a trolox equivalent (µmolTE/g) through DPPH method. Columns with same letter indicate no significant difference (P<0.05)

Figure 4.17 Average phenolic content (GAE) of mature greenhouse (GH) grown “Vrinda” plants compared to in vitro (IV) grown plants for root (R), stem (S) and leaf (L) analyzed via the FC method. Columns with same letter indicate no significant difference (P<0.05)
Figure 4.18 Quantification of melatonin concentration (ng/g) of leaves from greenhouse (GH) and field (F) plants with five biological replicates. Columns with same letter indicate no significant difference (P<0.05)

Figure 4.19 Quantification of melatonin concentration (ng/g) of roots from greenhouse (GH) and field (F) plants with five biological replicates. Columns with same letter indicate no significant difference (P<0.05)
Figure 4.20 Quantification of serotonin concentrations (ng/g) of leaves from greenhouse (GH) and field (F) plants with five biological replicates. Columns with same letter indicate no significant difference (P<0.05)

Figure 4.21 Quantification of serotonin concentration (ng/g) of roots from greenhouse (GH) and field (F) plants with five biological replicates. Columns with same letter indicate no significant difference (P<0.05)
Figure 4.22 Quantification of GABA concentrations (mg/g) of leaves from greenhouse (GH) and field (F) plants with five biological replicates. Columns with same letter indicate no significant difference (P<0.05)

Figure 4.23 Quantification of GABA concentrations (mg/g) of roots from greenhouse (GH) and field (F) plants with five biological replicates. Columns with same letter indicate no significant difference (P<0.05)
Figure 4.24 Leaves of three individual plants of the three varieties: “Tulsi”, “India” and “Garden” were analyzed for melatonin content (ng/g). Columns with same letter indicate no significant difference (P<0.05).

Figure 4.25 Leaves of three individual plants of the three varieties: “Tulsi”, “India” and “Garden” were analyzed for serotonin content (ng/g). Columns with same letter indicate no significant difference (P<0.05).
Table 4.1 Averaged concentrations in leaves and roots of GABA (mg/g), melatonin (ng/g) and serotonin (ng/g) of greenhouse and field grown plants with five biological replications. Columns with same letter indicate no significant difference between field and greenhouse (P<0.05)

<table>
<thead>
<tr>
<th></th>
<th>Leaf</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEL (ng/g)</td>
<td>SER (ng/g)</td>
</tr>
<tr>
<td>Field</td>
<td>327.17a</td>
<td>685.71a</td>
</tr>
<tr>
<td>Greenhouse</td>
<td>341.83a</td>
<td>497.95a</td>
</tr>
</tbody>
</table>
4.4 Discussion:

Holy basil is a well-known medicinal plant with many health benefits. The isolation and propagation of elite holy basil plants with high antioxidant content and significant quantities of other beneficial compounds such as melatonin, serotonin and neurotransmitter like GABA may be important for its medicinal use. In this study, we identified an elite accession of holy basil named “Vrinda” which was isolated based on its high antioxidant potential and presence of neurotransmitters including melatonin and serotonin.

4.4.1 Phenolic and Antioxidant Activity

High antioxidant activity is one of the major attributes for the medicinal value of holy basil (Mondal et al., 2009). Holy basil has high antioxidant value compared to other basil species (Trevisan et al., 2006) and is known as a medicinal plant in traditional texts. Holy basil improves wound healing in rats (Shetty et al., 2008), has a chemopreventative effects against many forms of cancer (Baliga et al., 2013; Gajula et al., 2009; N Singh et al., 2012), displays antidiabetic properties (Hussain et al., 2001), reduces peroxidative damage in liver and aortic tissues (Kedlaya and Vasudevan, 2004; Gupta et al., 2002; Geetha et al., 2004; Luthra, 2010), has neuroprotective effects after stroke (Yanpallewar et al., 2004; Ahmad et al., 2012) and reduces overall oxidative stress (Samson et al., 2007) all of these effects are attributed to the antioxidant activity of holy basil.

The aim of this study was to isolate elite accessions of holy basil with high antioxidant content. Seeds from five independent sources originating in both Canada and India were grown in vitro and individual plants were tested for antioxidant activity to
identify plants with high antioxidant potential. Seeds from the seed lot “Tulsi” from India was determined to have the highest antioxidant activity, with one plant labelled ‘T5’ having an antioxidant activity value of 1452.08 µmolTE/g of dry weight. Wangcharoen and Morasuk (2007) determined that the highest levels of antioxidant in white and red variety of holy basil were 5.41 and 6.23 µmolTE/g of dry weight, respectively. In another study multiple basil species were tested for antioxidant activity using the DPPH assay and a range of antioxidant activity from 199-547 µmolTE/mL of essential oil (EO) of 100g dry weight was determined among different species, with holy basil at 297 µmol TE/mL EO (Juliani and Simon, 2002). Based on the high antioxidant activity “T5” was isolated and was subsequently named “Vrinda”, one of many names of the goddess Tulsi.

Phytochemical variation between different genetic materials are common with medicinal plants and have been previously shown in holy basil (Gajula et al., 2009; Binns et al., 2002). Many factors can influence the development of secondary metabolites, including genetics and environmental factors (Ghasemzadeh and Ghasemzadeh, 2011). Gajula et al. (2009) compared the phenolic content of accessions of holy basil from Cuba, India and Denmark and found plants from Cuba had significantly higher levels of phenolics when compared to the other sources. Significant differences in the antioxidant activity were also present among these plants from different sources. This response is likely due to environmental and genetic variations.

Five individual “Vrinda” plants grown in the greenhouse were analyzed for their phenolic content and antioxidant activity. No significant difference was identified among
these plants indicating that there is little or no fluctuations in the medicinal activity among the plants vegetatively propagated from “Vrinda”.

The phenolic content of stems, leaves and roots of greenhouse and in vitro grown plants were compared the roots of the greenhouse grown plants had the highest phenolic content. These results are similar to the results of Shilpa et al. (2009), where the authors showed that the roots of the in vitro grown holy basil plants had higher antioxidant capacity than callus tissue. Additionally, the values reported here are comparable to the findings by Juliani and Simon (2002), where the authors found phenolic concentrations of various basil species ranging between 35.6 – 126.2 (mg GA/g dry weight) with holy basil being 51.1 GA/g. However, the values obtained by Wangcharooen and Morasuk (2007) were lower (12.60 – 19.46 GAEmg/g dry weight) than the leaf tissue of greenhouse grown “Vrinda” plants (60-95 GAE mg/g).

It is generally accepted that the leaf tissue of holy basil has the highest antioxidant value and therefore leaf tissue is the most medicinally important part of the plant (Engels and Brinckmann, 2013). A variation in environmental stress can affect the phenolic and antioxidant content of the plant (Nair et al., 2009). Higher antioxidant activity (6741.8 – 7717.6 µmolTE/g) was observed in “Vrinda” plants grown in the greenhouse compared to the in vitro grown plants (1452.08 µmolTE/g). Therefore, the variation between the plants grown in the greenhouse and in vitro is likely due to the environmental differences.

Additionally, plants grown in field conditions appeared purple in the stem and leaf while indoor grown plants remained green. Using the flower, leaf and stem samples,
they were analyzed for antioxidant potential, with no significant difference between the organs tested (leaf, flower and stem) or in growth conditions. The lack of variation observed here indicates that the antioxidant activity of the plant does not vary in different growing conditions. In contrast, previously using the two varieties of holy basil, purple and green, have been reported to have increased activity in the purple plants (Wangcharoen and Morasuk, 2007; Juliani and Simon, 2002). These variations previously seen are likely due to cultivar variation, rather than using clonally propagated genetic material.

4.4.2 Melatonin, Serotonin and GABA Concentrations in Holy Basil

The elite plant “Vrinda” contains levels of melatonin within the leaf and root tissue at average concentrations of 334.5 ng/g in the leaf and 371.8 ng/g in the root. In comparison, other plants known to contain melatonin showed a range of concentrations: with St. John’s Wort at about 4 ug/g tissue (Murch et al., 1997), cereals such as corn, rice and barley at about 2 ng/g , and ginger at 1.4 ng/g tissue (Badria, 2002). High levels (up to 1000ng/g) of melatonin was also detected in some Chinese medicinal herbs (Chen et al., 2003). Chinese medicinal herbs including Agastache rugosa, Scrophulariae ningpoensis, Rosaceae chingii and Syringa yunnanensis contain melatonin levels similar to that identified in holy basil (Chen et al., 2003). Melatonin has been proven to be related to plant adapting to stress, however, melatonin may also have auxin like effects, which may explain the increased levels found in root tissue (Tan et al., 2012).
Serotonin, the precursor to melatonin, is a well-known neurotransmitter in humans, and has been associated with a range of health benefits. Levels of serotonin was observed in holy basil leaves (127.6 – 1205.0 ng/g) and roots (153.3 - 716.6 ng/g). These levels are lower to the levels observed by Badria (2002) in some food and medicinal plants. A variety of medicinal plants were previously shown to contain higher serotonin to melatonin levels (Badria, 2002; Murch et al., 2009)

GABA is another common human inhibitory neurotransmitter (Chebib and Johnston, 1999). In plants, the regulation of GABA is affected by stress conditions, nitrogen and carbon metabolism, oxidative stress protection, pH regulation, and is involved in defense mechanism against herbivores, osmoregulation and plant signalling (Bouché and Fromm, 2004; Roberts, 2007; Fait et al., 2008; Stafford, 2005; Shelp et al., 1999). In the analysis of field and greenhouse grown root and leaf tissue, no significant difference was observed in GABA levels. Higher concentrations of GABA were present in leaf tissues compared to the roots. This variation could be explained due to the higher abiotic and biotic stress experienced by leaves compared to roots.

The higher concentration of antioxidants and neurotransmitters, melatonin, serotonin and GABA detected in holy basil leaves are some of the probable reasons for its healing properties. An increased level of melatonin in humans has been observed with ingesting melatonin rich products such as coffee (Ursing et al., 2003), vegetables (Oba et al., 2008), and grains (Tan et al., 2012). These chemicals are well recognised for their health benefits associated with antioxidant properties, improvement of sleep disorders, decreased instances of migraines, epilepsy, hypertension, irritable bowel
syndrome, platelet function, and mood disorders (Mohammad-Zadeh et al., 2008; Stafford, 2005; Reiter and Tan, 2001).

4.5 Conclusion:

Eighty accessions of holy basil were analyzed from different sources for their antioxidant potential. Of these plants, an individual plant with high antioxidant potential, “Vrinda”, was selected and clonally propagated. The growth habits of “Vrinda” plants were studied in vitro, in the greenhouse and in field conditions. The “Vrinda” plants grown in the greenhouse and field had higher antioxidant potential than the in vitro grown plants. This may be due to the increase in abiotic and biotic stress faced by the plants outside. It was also demonstrated that clonally propagated plants grown in vitro have no significant difference in their phenolic and antioxidant capacity when transferred into greenhouse. Little variation between growing conditions suggests that plants can be propagated with consistent active ingredients, a key requirement for the process of producing NHPs. Melatonin, serotonin and GABA, well known for their health benefits in humans, were identified in these plants. Further research on consumption of holy basil and the influence these neurotransmitters on human health are needed to determine if these compounds contribute to the medicinal activity of holy basil.
5.0 Chapter 5: Morphological Analysis of the Elite Holy basil

(*Ocimum sanctum*) accession “Vrinda”

Abstract:

An elite accession of holy basil, “Vrinda”, was identified based on its high antioxidant potential and phenolic content. To assess its agronomic characteristics for large scale production, plants were grown in greenhouse and field conditions and compared to another population of holy basil. The plants had similar growth patterns, but “Vrinda” plants had a delayed flowering response. “Vrinda” plants had deep purple leaves, stems, and inflorescence when grown in the field, but were green when cultivated in the greenhouse. Scanning electron microscopy of leaves and petioles was used to investigate trichome characteristics of the plants. No significant variations in trichome size or density were found between plants grown in both condition. Three types of trichomes common to the Lamiaceae family were identified in leaves of “Vrinda”. Overall, in addition to high antioxidant potential and phenolic content, “Vrinda” plants displayed good agronomic characteristics for commercial production.

5.1 Introduction:

In the natural health product industry, *in vitro* propagation is a useful technique to maintain and multiply germplasm selected for high medicinal value (Paek et al., 2005; Rout et al., 2000), and helps to comply with regulations and guidelines put in place by Health Canada regarding quality and purity standards (Government of Canada, 2004e). For a continuous supply of high quality holy basil, mass production through *in vitro* propagation combined with greenhouse production would be an ideal system. A similar
approach has been explored for a number of other medicinal plants (Murch and Saxena, 2006; Thatoi and Patra, 2011; Debnath et al., 2006). In order to develop a commercially viable production system, plants with increased phytochemical content combined with suitable agronomic characteristics are needed.

Few reports investigating morphological and agronomic characteristics of *Ocimum sanctum* have been reported (Jürges et al., 2009; Wetzel et al., 2002). In general, holy basil plants grow 30-150cm tall, have hairy stems and leaves, with simple ovate or elliptical opposite leaves that can be entire or serrate. Both the stem and leaves can range from purple to green in colour, with flowers showing a range of purple. In previous studies (Jürges et al., 2009; Wetzel et al., 2002), greenhouse and field grown holy basil was characterized and compared to other *Ocimum* species for micro and macro morphology, but were only characterized at full maturity. Morphological characterization from the seed germination to maturity stage is important to understand whole growth cycle. Information for seed germination, seedling growth, vegetative growth and reproductive phase are required to characterize elite lines and determine if they are suitable for commercial production. Microclimates also affect plant growth, development, and chemical properties. It is better to characterize plants in control environmental conditions and in open fields to determine these effects.

Microscopic leaf analysis has been done previously on holy basil (Jürges et al., 2009) and described trichome structure for identification purposes. Trichomes are found on almost all above ground tissues. In the family Lamiaceae, there are two major groups of trichomes; glandular trichomes which are secretory structures that store and excrete exudates, while non-glandular trichomes are hair-like structures that do not
accumulate secondary metabolites (Wagner, 1991; Hutzler and Fischbach, 1998). Trichome characteristics are essential for identification purpose and indirectly represent its potential medicinal value.

In the previous chapters, establishment of an efficient micropropagation method for holy basil was established and an elite accession was identified based on high antioxidant potential and phenolic content. The goal of this study was to characterize the morphology and agronomic characteristics of the plant population of “Vrinda” to ensure its quality for production in the greenhouse or field. Selfed seeds were collected from a population that had a consistent antioxidant activity as seen in previous chapters (Chapter 3). The main objective of these experiments was to determine the morphological characteristics in comparison to another holy basil plant population in greenhouse conditions. Secondly, a study was conducted to examine phenotypic variation and trichome differences of “Vrinda” population growing in both greenhouse and field conditions.

5.2 Materials and Methods:

5.2.1 Growth and development characteristics of plants:

Seed were collected from selected “Vrinda” plants that were selfed in isolated greenhouse rooms and compared to seeds from a seed lot from India, named “Garden”. Seeds were germinated in 16 well greenhouse trays using Sunshine Soilless potting mixture number 4 in the greenhouse. Germination percentages were recorded after 8 days of sowing. Four week old seedling were transferred to 5” pots with the same potting mix composition and returned to the same greenhouse conditions. A total of 18
plants of each population were selected and divided into three groups. Each group was spatially separated on different benches of the greenhouse in a completely randomized design.

All plants were assessed for height (mm), number of internode, number of axillary bud, leaf number, percentage of plants flowering, leaf and stem colour on a weekly basis. Three random leaves were removed and scanned to assess leaf area (cm²) using ImageJ 1.47v (National Institute of Health, USA). Leaves and axillary buds were counted when fully expanded. Observations were recorded for 8 weeks after transplanting. After eight weeks, plants were removed from the pots and observations were recorded for root growth.

5.2.2 Comparison of “Vrinda” plant population in different growth condition:

Plants from “Vrinda” seed were grown in field condition during the months of May to August and compared with greenhouse population. Conditions of the greenhouse were 24°C at 16 h light and 20°C for 8 h darkness. Plants were assessed for visual variation in physical changes including colour and differences in physical appearance for stem, leaf and inflorescences.

5.2.3 Comparison of Trichomes:

Three leaves and petioles from three plants were collected from the greenhouse and field grown “Vrinda” plants to compare trichome development. Tissues were imaged using an S-570 SEM (Tokyo, Japan) with Quartz PCI software (Quartz Imaging Corp., Vancouver, Canada). Images were analyzed using ImageJ 1.47v (National Institute of Health, USA) to determine trichome density, glandular trichome diameter, and area. For
the density of trichomes, three 500 x 500 µm areas were randomly selected and the number of hairy, capitate glandular and peltate glandular trichomes was counted. The diameter of three randomly selected large and small trichomes were measured using the longest distance across the structure and averaged for each treatment and tissue type. Comparisons of the density, diameter, and areas of the three types of trichomes between the adaxial, abaxial leaf side and petiole of each treatment were completed.

5.2.4 Statistical Analysis:

All the experiments were conducted using a completely randomized design and analysis of variance was conducted using JMP version 11 for Windows (SAS Institute Inc. Cary, NC). Data was pooled after no significance difference was observed between the three replications and the two means were compared using a t-test (P<0.05). Means of trichomes statistics were compared using Tukey’s test (P< 0.05). The data are presented as means ± standard error of means.

5.3 Results:

5.3.1 Growth and development behaviour of “Vrinda” seeds

Germination percentages were significantly higher with the “Vrinda” plants at an average of 70% when compared to the “Garden” at 53.75% (Figure 5.1). The number of leaves increased over the period of eight weeks, however average leaf area decreased in both plant populations (Figures 5.2, 5.7). Higher numbers of leaves were observed in “Garden” plants compared to “Vrinda” throughout the growth period, however significant differences were observed during weeks six and seven. Leaf area was not significantly
different between “Garden” and “Vrinda” plants during the growth period except for week 12 (Figures 5.7, 5.11).

Results demonstrated that the average number of axillary buds was not significantly different between the populations during the initial growth period up-to nine weeks with the exception of week of seven. The difference was significant later in the growth period (Figures 5.3, 5.12). A significant difference was observed for number of axillary buds between “Garden” and “Vrinda” for the growth in week 10, 11 and 12. The average number of axillary buds observed in “Vrinda” was 39 at the end of 12th week of growth, while in “Garden” it was 101.

“Vrinda” plants showed an average of 11 internodes and a plant height of 391.3mm at the end of 12 weeks. This was similar to the “Garden” plants (Figures 5.4 and 5.6). “Vrinda” plants were significantly shorter than “Garden” plants at week eight with average plant height of “Vrinda” plants at 292.0 mm and “Garden” plants measuring 384.5 mm.

Observations for flower initiation revealed later flowering in “Vrinda” plants, nearly 84 days after germination. Percentage of flowering among “Garden” was earlier than “Vrinda” (Figure 5.5). “Garden” had two-thirds of the plants flowering at 56 days after germination, whereas at that point only 17% of “Vrinda” plants had begun to flower. By week nine (63 days), nearly all the “Garden” plants had begun to flower, while the majority of “Vrinda” plants were still in the vegetative phase at eleven weeks (77 days) of observations.
Colour differences were apparent early in the measurements. “Garden” and “Vrinda” plants had green stems and leaves at week five (Figure 5.8). By week six, the stems of “Garden” plants began to change from green to purple (Figure 5.9) and by week seven of observations, dark purple colouration of the stem was apparent (Figure 5.10). This transition to purple in the stem was not apparent in “Vrinda” until week nine of observations (Figure 5.12). By week nine, inflorescences were present on the majority of the “Garden” plants (Figure 5.13) while only one plant had begun to flower in “Vrinda”.

Root structure was observed after 12 weeks to observe if any differences were present. “Garden” had short, thin and tightly compact roots, with the longest root measuring 190 mm, “Vrinda” plants had a long primary root (300 mm) with fleshy roots at the crown (Figure 5.14, 5.15).
Figure 5.1 Germination percentage of seeds from “Vrinda” and “Garden” grown in greenhouse conditions. Columns with same letter indicate no significant difference (P<0.05)

Figure 5.2 Average number of leaves for greenhouse grown plants, “Vrinda” and “Garden”, after four weeks of germination to 12 weeks of growth. Data points with (*) indicate significant difference within that week (P<0.05)
Figure 5.3 Average number of axillary buds on greenhouse grown plants, “Vrinda” and “Garden” between five and 12 weeks of growth. Data points with (*) indicate significant difference within that week (P<0.05)

Figure 5.4 Average number of internodes on greenhouse grown plants, “Vrinda” and “Garden” between five and 12 weeks of growth. Data points with (*) indicate significant difference within that week (P<0.05)
Figure 5.5 Flowering percentages from week five to 12 in greenhouse conditions. Data points with (*) indicate significant difference within that week (P<0.05)

Figure 5.6 Average height (mm) of greenhouse grown plants, “Vrinda” and “Garden” between five and 12 weeks of growth. Data points with (*) indicate significant difference within that week (P<0.05)
**Figure 5.7** Average leaf area (cm$^2$) of greenhouse grown plants, “Vrinda” and “Garden” between five and 12 weeks of growth. Data points with (*) indicate significant difference within that week (P<0.05)

**Figure 5.8** Holy basil plants (A) “Vrinda” and (B) “Garden” growing in greenhouse conditions after five weeks of germination
Figure 5.9 Holy basil plants (A) “Vrinda” and (B) “Garden” growing in greenhouse conditions showing color variation in stem after six weeks of germination
Figure 5.10 Holy basil plants (A) “Vrinda” and (B) “Garden” growing in greenhouse conditions showing color variation in stem and axillary branching after seven weeks of germination
Figure 5.11 “Garden” plants showing (A) purple flower and (B) dark purple stem after ten weeks of germination growing in greenhouse condition. Similarly, “Vrinda” plants showing (C) purple stem and (D) stem color transiting from green to purple after ten weeks of germination growing in greenhouse condition.
Figure 5.12 (A) “Vrinda” plants showing compact axillary branching compared to (B) “Garden” plants with axillary branching after 11 weeks of germination growing in greenhouse condition.
Figure 5.13 (A) “Vrinda” plants showing compact plant structure compared to (B) “Garden” plants with spaced axillary branching with multiple inflorescences after 12 weeks of germination growing in greenhouse condition.
Figure 5.14 Holy basil plants grown in greenhouse conditions demonstrating the variation in root development after 12 weeks after germination. (A) “Garden” and (B) “Vrinda”

Figure 5.15 Holy basil plants grown in greenhouse conditions demonstrating the variation in root development after 12 weeks after germination. (A) “Garden” and (B) “Vrinda”
5.3.2 Comparison of greenhouse and field grown “Vrinda” plants:

Variation in size and color for leaves, stem and inflorescences were observed for the “Vrinda” population growing in green house and field condition. Greenhouse grown plants had green leaves on both adaxial and abaxial sides, inflorescence with light purple flowers and green stems (Figures 5.16, 5.17, 5.18). However, the adaxial sides of leaves, stem, inflorescence and flowers of the field grown plants displayed dark purple colouration (Figures 5.16AC, 5.17A). Similar to greenhouse grown plants, the abaxial side of the leaf exhibited green color (Figures 5.14BD)

Leaves of field grown plants had an oval shape with serrated margins whereas the greenhouse grown leaves displayed elliptic with entire margins (Figs. 5.15, 5.16). Both plants demonstrate opposite leaf arrangement with racemes forming at the terminal end of the stem. Flowers were in closed whorls (Figure 5.18).

5.3.3 Comparison of Trichome Development for greenhouse and field grown “Vrinda” plants:

Leaf tissue collected from field grown plants was purple, while from greenhouse grown plants was green. Petioles from greenhouse and field grown plants showed glandular and non-glandular trichomes, however no significant differences were observed for average numbers of hairy trichomes (Figures 5.19, 5.27). There were no significant differences for peltate glandular trichomes average number, area and diameter in both samples (Figures 5.22, 5.23, 5.26). In the case of capitate glandular trichomes on the petiole, no significant differences were observed for the average number and diameter (Figures 5.24, 5.28) but petiole from greenhouse plants (894.46
µm²) showed significant difference for area as compared to field grown plants (481.22 µm²).

The number, area and diameter of glandular and non-glandular trichomes were assessed on the abaxial side of field and greenhouse grown “Vrinda” leaves (Figure 5.20). There were significantly more hairy, non-glandular trichomes (13) in leaves from field grown plants than the greener leaves of greenhouse grown plants (4) (Figure 5.27). Peltate glandular trichomes were not significantly different in density number or area (Figures 5.23, 5.26). Peltate trichomes on the field grown leaves were significantly different in diameter (61.03 µm) than the greenhouse grown plants (46.92 µm) (Figure 5.23). Capitate trichomes showed no significant difference in density, area and diameter (Figures 5.24, 5.25, 5.28).

The adaxial sides of both greenhouse and field grown leaf tissue were examined for glandular and non-glandular trichomes for average number, area and diameter (Figure 5.19). The average number of hairy was higher in field grown tissues (10) compared to greenhouse grown plants (3) (Figure 5.27). There were no significant differences for area and diameter for capitate trichomes (Figures 5.24, 5.25). Peltate glandular trichomes had no significant differences in number or diameter (Figures 5.23, 5.27), but their average area was significantly higher in field grown leaves (2865.46 µM²) than greenhouse leaves (1746.85 µM²) (Figure 5.24).
Figure 5.16 Leaf comparison of (A) adaxial and (B) abaxial side of leaf tissue. (C) Indicates field grown “Vrinda” plants while (D) indicates greenhouse grown “Vrinda” plants.

Figure 5.17 Comparison of stem, leaf, and inflorescence of (A) field grown “Vrinda” plants with (B) greenhouse grown “Vrinda” plants.
Figure 5.18 Comparison of inflorescence of (A) field grown “Vrinda” plants (B) greenhouse grown “Vrinda” plants at maturity
Figure 5.19 Scanning electron micrographs of petioles from mature holy basil, “Vrinda”, grown in greenhouse conditions (A and B) and field conditions (C and D) captured for structure and variation of trichome formation (Scale ranges from 50 – 500 µm)
Figure 5.20 Leaves from mature holy basil, “Vrinda”, grown in greenhouse conditions with green leaves (A and B) and field with purple leaves (C and D) captured for structure and variation on the abaxial side of the leaf tissue using scanning electron microscope.
Figure 5.21 Leaves from mature holy basil, “Vrinda”, grown in greenhouse conditions with green leaves (A and B) field with purple leaves (C and D) captured for structure and variation on the adaxial side of the leaf tissue using scanning electron micrograph (Scale ranges from 50 – 100 μm)
Figure 5.22 Average diameter (µm) of peltate glandular trichomes found on the adaxial and abaxial leaf and petiole of greenhouse and field grown “Vrinda”. Columns with same letter indicate no significant difference (P<0.05)

Figure 5.23 Average area (µm²) of peltate glandular trichomes found on the adaxial and abaxial leaf and petiole of greenhouse and field grown “Vrinda”. Columns with same letter indicate no significant difference (P<0.05)
**Figure 5.24** Average diameter (µm) of capitate glandular trichomes found on the adaxial and abaxial leaf and petiole of greenhouse and field grown “Vrinda”. Columns with same letter indicate no significant difference (P<0.05).

**Figure 5.25** Average area (µm$^2$) of capitate glandular trichomes found on the adaxial and abaxial leaf and petiole of greenhouse and field grown “Vrinda”. Columns with same letter indicate no significant difference (P<0.05).
Figure 5.26 Average number of peltate glandular trichomes found in a 500 µm$^2$ area on the adaxial and abaxial leaf and petiole of greenhouse and field grown “Vrinda”. Columns with same letter indicate no significant difference (P<0.05)

Figure 5.27 Average number of non-glandular hairy trichomes found in a 500µm$^2$ area on the adaxial and abaxial leaf and petiole of greenhouse and field “Vrinda”. Columns with same letter indicate no significant difference (P<0.05)
Figure 5.28 Average number of capitate glandular trichomes found in a 500 µm² area on the adaxial and abaxial leaf and petiole of greenhouse (green) and field “Vrinda”. Columns with same letter indicate no significant difference (P<0.05)
In comparing adaxial, abaxial and petioles within either the greenhouse or field plants some trends arise. Peltate trichomes in greenhouse tissue were not significantly different in average diameter and number. Peltate trichomes on the petiole were significantly larger than the adaxial side of the leaf. Peltate trichomes in all field grown tissues were not significantly different for average number, diameter or area. Capitate trichomes in the field grown tissue were not significantly different in average area or diameter. Significantly more trichomes were found on the abaxial side of the leaf than the adaxial side or petiole of field tissue and all greenhouse tissue. Greenhouse grown leaves and petioles also had no significant difference in the diameter or number of capitate trichomes, but the average area of the petiole was significantly less than the abaxial side or adaxial side of the greenhouse leaf. The average number of hairy trichomes on the petiole of greenhouse grown tissue was significantly greater than adaxial or abaxial greenhouse leaf, similar to the field grown tissue hairy trichomes were significantly higher in the petiole than the adaxial side of the leaf.

5.4 Discussion:

For mass production of medicinal plants the use of greenhouse controlled environments can assist in increasing biomass, reduce risk of contamination, improve consistency and increase levels of secondary metabolites (Fonseca et al., 2006). Therefore, characterizing greenhouse growth behaviours and morphological variations is required for production of a natural health product to increase harvest.

In this experiment, the objective was to compare the morphological differences of growth and development of the elite plant population of “Vrinda” to another seed lot,
“Garden” (Chapter 3). The population “Vrinda” had various factors evaluated to determine the morphological characteristics. Additionally, plants of “Vrinda” in both field and greenhouse conditions were compared for physiological differences and for trichome variations.

5.4.1 Greenhouse Comparison of “Garden” and “Vrinda”:

“Vrinda” germination rates in the greenhouse were much higher than what was found in Chapter 1 in vitro. Germination rates in the literature are described as poor (Tyub and Kamili, 2009; Pattnaik and Chand, 1996). In the present study, germination percentages were 70% for “Vrinda” and 53.7% in “Garden”, however, the seeds collected from “Vrinda” were newer which may have contributed to the higher germination rate. Holy basil is conventionally grown from seed, with no form of vegetative propagation commercially practiced (Pattnaik and Chand, 1996). Higher germination rates for conventional propagation methods are important for improving yield and success of the population.

Generally, there were not many significant differences in the growth characteristics of the two plant populations. “Garden” was overall larger and had an increased value for all significant differences in almost all of the parameters analyzed. “Vrinda” was visibly smaller and less bushy, but not significantly different than “Garden” for most weeks assessed.

At early time points, there were some significant differences in axillary bud number (week 7), height (week 8) and leaf number (weeks 6 and 7), favouring “Garden” over “Vrinda”. These differences could be attributed to timing of flowering. By seven
weeks after germination, 17% of the plants had begun to flower in “Garden”, while no flowering was apparent in “Vrinda”. This observation is in similar with information that reports flowering at 8-10 weeks in holy basil (Darrah, 1974). The authors also indicated plants need to be between 25 cm-38 cm before flowering occurs (Darrah, 1974). By week six, “Garden” had reached an average height of 25 cm, and in the next week flowering had begun. This height was not reached until week seven in “Vrinda”, and in the following week an individual plant had begun to flower. For “Vrinda”, the flowering response was not observed until weeks 11 and 12 (33% and 66%, respectively), where plants were within the peak 38 cm height range on average. This is in agreement with previous observations regarding the height requirements prior to flowering. This information is useful as delayed flowering and maintaining shorter plants may increase the biomass of vegetative tissue.

Increased production of leaves prior to flowering of “Garden” (week six and seven) were likely preparing the plants for the energy demanding transition to reproductive stage (Lang, 1952). By week eight, 67% of “Garden” plants were flowering. Between 9-11 weeks, “Garden” had significantly higher flowering percentages than Vrinda.

Delayed flowering can lead to an increase in vegetative growth, which may potentially lead to an increase in number of harvests over the year. After 84 days, only 33% of “Vrinda” had begun to flower compared to 100% of “Garden” plants. A previous report on holy basil reported 98 days to flower outdoors (Simon et al., 1999). The decrease in time could be attributed to the controlled environmental conditions that greenhouse provides rather than the variability found in the field.
Visually, plants had similar morphological appearances; however, “Garden” shows early darkening of the stem in the greenhouse compared to “Vrinda”, which remained green over time. “Vrinda” also had a visually compact appearance when compared to “Garden” which had increased branching and terminal flowers on numerous branches, especially apparent in the last three weeks of observation. “Vrinda” appeared to have only an individual inflorescence that developed at the apical meristem rather than axillary branches. This compact appearance would be helpful for developing a commercial product. Plants may also be more desirable when they are smaller in appearance to decrease spacing between plants in an area and for ornamental purposes in consumers’ homes.

The average height was 391 mm in “Vrinda” and 475 mm for “Garden” at week twelve, which were not significantly different. A study by Jürges, Beyerle, Tossenberger, Häser, and Nick (2009) analyzed the morphology of different accessions of holy basil grown in greenhouses. In their characterization they describe the plants as reaching 30-50cm in height. *O. sanctum* was studied in field conditions and characterized a variety of physiological traits. Mean height of the plants was 34.25cm, with a maximum height of 57cm (Wetzel et al., 2002).

Comparisons of “Vrinda” and “Garden” holy basil plants in this study determined that overall the plants were not significantly different in the morphological appearance. Visual observations demonstrate that “Vrinda” plants were smaller, more compact with a longer period of vegetative growth before transitioning to the reproductive stage.

5.4.2 Comparison of Field and Greenhouse Grown “Vrinda” Plants:
“Vrinda” grown in field conditions showed a drastic effect on colour of the stem, leaf and inflorescence. It is known that there are different varieties of Tulsi, Krishna and Rama which are purple or green, respectively (Engels and Brinckmann, 2013). In this study, the “Vrinda” plant population had a variation of colour that was observed based on growing conditions. For the plants in the greenhouse, the entire plant was green with minimal purple colouration of the flower and stem. In comparison, the field grown plants were deep reddish-purple except for the abaxial side of the leaf, which remained the same colour as the greenhouse grown plants.

Plants produce pigments to improve photosynthetic capacity in low light conditions, but plants also produce these pigments to protect from ultraviolet light (UV) damage (Teramura and Sullivan, 1994). Ultraviolet light, specifically UV-B, has been shown to damage plants; impacting photosynthesis and overall growth, causing oxidative damage to tissues and decreasing function of the plant (Teramura and Sullivan, 1994; Ambasht and Agrawal, 1995; Zlatev et al., 2012). To combat this stress, plants alter their leaf morphology and synthesize compounds such as flavonoids and phenolics to minimize damage. One specific flavonoid is the category of pigments, anthocyanins (Chalker-Scott, 1999). These pigments are part of the phenolic shikimate pathway found in fruit, flowers and leaf tissue to give blue, red and purple colouration (Chalker-Scott, 1999). As the plants grown in greenhouse conditions are shielded from UV light by the glass greenhouse (Teramura and Sullivan, 1994) the UV light in the field growth condition is likely to contribute to the increase in pigments found in the plants. Exposure of plants to UV-B light has been previously shown to increase pigment and antioxidant production in aerial tissue (Zlatev et al., 2012).
In red leaf lettuce, reducing exposure of UV-B also reduced the flavonoid and anthocyanin production (Krizek et al., 1998). Alternatively, in corn, the addition of UV-B light increased anthocyanin production (Ambasht and Agrawal, 1995), and in snow algae a 5-24% increase in phenolic production was found with exposure to UV-B light (Duval et al., 1999). Wellmann (1975) and Liu et al., (1995) both observed this response in barley, where an increase in flavonoid production was associated with UV-B light exposure. Additionally Liu et al., (1995) discuss another phenolic acid, hydrocinnamic acid, absorbing UV-B radiation similar to that of flavonoids. Wellmann (1975) also observed an increase in the PAL pathway, one of the first steps of phenolic synthesis. This was also observed with cabbage plants treated with a PAL inhibitor, which lead to an increase in UV-B damage due to the decrease in phenolic and flavonoid production (Gitz et al., 2004). The fact that the abaxial side of the leaf is still green after growing outdoors further implies that the UV light could be the major cause of the variation in colouration.

5.4.3 Trichome Development between Field and Greenhouse “Vrina” Plants:

Three types of trichomes were apparent on the petiole and leaf lamina. Two glandular trichomes were identified, the first, a four lobed larger trichome (peltate) and the second, a smaller uni- or bicellular lobed trichome (capitate). A non-glandular, unbranched hairy trichome was also identified on the leaves and petioles. The average diameter and area of the glandular trichomes were determined, and average number per 500µm² of all three types of trichomes was assessed.
The non-glandular hairy trichomes were denser on the petioles of the field when compared to the adaxial side of the field leaf. Furthermore, the field leaf tissue had more of hairy trichomes on the petiole, adaxial and abaxial side of the leaf when compared to the greenhouse grown green leaves. Hairy trichomes are non-glandular and do not secrete secondary metabolites (Wagner, 1991). Their hypothesized function is to protect the leaf from predators and help to moderate leaf temperature, water loss and sunlight absorption (Wagner, 1991; Hutzler and Fischbach, 1998), and to protect the leaf from UV-B damage (Karabourniotis et al., 1995). Because of these functions it is likely that the field grown leaves would require more hairy trichomes as they are more prone to abiotic and biotic stress than plants in environmentally controlled greenhouse conditions.

The two types of glandular trichomes were apparent on all tissue types. Glandular trichomes are known to be primary secretory organs, as they compartmentalize phenylpropanoids and terpenoids including hydrocinnamic acids, coumarins, stilbenes and styrl pyrones (Hutzler and Fischbach, 1998).

Generally there is a lot of variation in trichome structure between plant families, and within the family Lamiaceae it is common for plants to have both or either peltate and capitate trichomes present (Marin et al., 2010, 2008; Baran et al., 2010; Wogiatzi et al., 2011; Gersbach, 2002; Venkatachalam et al., 1984). Morphology of their trichomes is variable between species with different numbers of lobes, stalk height or basal cells. Generally capitate trichomes are stalked with a unicellular head, while peltate trichomes have a stalk that is short and embedded into the epidermis and has a head with numerous lobes (Venkatachalam et al., 1984). Peltate trichomes on holy basil are
shown to have four lobes, and are larger than the capitate with either uni- or bicellular lobes and a single celled stalk. Holy basil peltate trichomes are depressed into the epidermis of the leaf tissue, as is similarly shown within the family with salvia (Venkatachalam et al., 1984; Baran et al., 2010); Satureja subspicata (Marin et al., 2010); thyme (Marin et al., 2008); and mint (Gersbach, 2002).

Few significant differences between the greenhouse and field glandular trichome morphologies were observed. The abaxial side of the leaves from field grown plants had significantly more capitate trichomes than the greenhouse abaxial side of the leaf, although the diameter of capitate trichomes did not vary. This would be expected as the leaf in the field environment is exposed to many stresses than the controlled environment in the greenhouse, requiring the use of antioxidants and other defense compounds to protect it. An increase in this density has been observed in other species when exposed to UV light (Kostina et al., 2001; Karabourniotis et al., 1995). The area of capitate trichomes was significantly smaller in the field leaf and petiole when compared to the greenhouse petiole and abaxial side of the leaf. This could indicate that although the density of the trichomes was higher in the field leaf, this may have been due to smaller sized trichomes. Potentially a benefit for the plant as stress factors could damage the leaf or trichomes. By having more individual trichomes, leaves may not be as detrimentally compromised by damage.

Peltate trichomes were not as dense on the leaf or petiole as the capitate trichomes, and there were no significant differences among tissues. The diameter and area between the two treatments were not significantly different in most instances,
indicating little variation in the peltate trichome development between field and greenhouse varieties of holy basil.

This is the first time trichome structure comparing field and greenhouse leaves and petioles of holy basil has been done. Previously, Jürges et al., (2009) observed holy basil leaves and confirmed the presence of glandular hairs on both sides of the leaf surface. They identified smaller glandular hairs with two cells. In this present study the identification of larger, peltate trichomes with four lobes on “Vrinda” was observed. Previous work in the family Lamiaceae confirms the presence of hairy non-glandular trichomes, peltate and capitate glandular trichomes are a common occurrence. Morphology of these trichomes varies slightly within each species in number of lobes or stalk height of both peltate and capitate trichomes. Trichome development varies little between the two conditions with all types of trichomes found on the abaxial and adaxial side of the leaf and petiole. This coincides with the consistency in antioxidant production between the two treatments previously observed (Chapter 3).

5.5 Conclusions:

In this study, the aim was to characterize growth patterns and morphology of the holy basil plant population of “Vrinda” on a weekly basis along with comparisons of mature plants of field and greenhouse grown plants. This characterization is beneficial for the introduction of the plant in greenhouse conditions for developing a standardized natural health product and improving the yield and frequency of harvest. Analysis of the morphology of the elite accession “Vrinda” shows a few significant differences compared to other sources of holy basil. Growing plants in field conditions initiates
pigment formation in the leaf, stem and inflorescence and is likely due to increased UV-B radiation but had little effect on trichome development.

The characterization of “Vrinda” over time has shown compact plants, with delayed flowering time compared to another seed source. Field conditions increase pigment production over plants grown in the greenhouse, likely due to high stress environment. These desirable traits indicate “Vrinda” as a potential population for traditional cultivation methods for generating natural health products from holy basil.
Holy basil (*Ocimum sanctum* L.) is a sacred herb used in ancient medicinal practices. The plant, native to Asia, is a species of basil, with aromatic leaves that range from purple to green in colour (Engels and Brinckmann, 2013). The plant has been found to assist in numerous ailments ranging from acute to chronic, and has long term health benefits with constant use (Das and Vasudevan, 2006; Mondal et al., 2009). Holy basil has been used for centuries because of the plants extensive medicinal properties, including antimicrobial, adaptogenic, anticancer, antidiabetic and antistress amongst many other beneficial traits (Bathala et al., 2012; Baliga et al., 2013; Viyoch et al., 2006; Gupta et al., 2007; Rai et al., 1997). Due to this range of uses to treat and prevent disease, the use of holy basil as a natural health product (NHP) in Western society may be an asset to the populations’ long term health. In Canada, there are strict rules and regulations for NHPs to ensure high quality products (Government of Canada, 2004e); however, there are still instances of contamination of products currently on the market (Newmaster et al., 2013).

Use of micropropagation for holy basil may help alleviate some of these risks with NHPs by establishing cultures with high medicinal value in sterile controlled environment systems. Micropropagation of holy basil has been investigated previously, but an efficient production protocol has not been developed. Not only does micropropagation assist with production of plants, but also allows for the maintenance of large populations that can be further analyzed for their medicinal value (Rout et al., 2000). The main constitutes in holy basil believed to give it such widespread capabilities is the numerous phenolic compounds found in the leaf tissue (Mondal et al., 2009).
These secondary metabolites are thought to be used by plants as antioxidants to protect and defend against stress. In humans, these secondary metabolites have a range of pharmacological activities improving overall health (Ghasemzadeh and Ghasemzadeh, 2011).

The objectives of this thesis were to develop a micropropagation system for holy basil and to identify elite plants to aid in the development of a domestic holy basil production system in Canada. To establish a micropropagation protocol for holy basil, shoots were initiated and multiplied in vitro. This was achieved with a high success rate using surface sterilization and media containing BA 1.1 µM and GA3 0.3 µM (Chapter 2). Using this protocol, plants had improved multiplication rates and good shoot development. Plants were transferred into rooting medium which induced root formation in all treatments, but the use of IBA at 0.5 µM in combination with activated charcoal (0.6%) was found to be optimal. Transferring plants out of tissue culture was successful with a survival rate of 83%. Plants were slowly acclimatized from the tissue culture environment of high humidity and low light using misting beds. While plants grew and multiplied well in this system, further improvements were needed in multiplication rates and the frequency of subculturing due to destabilization of the solidifying agent.

Further comparisons with the use of additional media additives in holy basil culture systems were conducted to improve multiplication and increase efficiency. Investigations into different gelling agents, pH buffers and liquid state medium decreased growth and multiplication, and many died (Chapter 3). The use of activated charcoal is known to improve in vitro cultures through many mechanisms, including providing dark environments, stabilizing pH, adsorbing harmful compounds and
releasing compounds that promote growth (Pan and Staden, 1998). Addition of 0.6% activated charcoal in the multiplication culture media improves the multiplication rate from 2.5 shoots per explant in the previously established, to nearly 4 shoots per explant. Activated charcoal also improved the efficiency of the process by extending the length of time between subculturings and improving the stability of the solidified media.

Additionally, the use of AIP, an inhibitor of phenolic biosynthesis (Appert et al., 2003), further improved multiplication rates and shoot development at 2 µM when combined with activated charcoal. These data suggest that holy basil produce phenolic compounds that inhibit growth and development in vitro. AIP improved shoot growth and the low levels did not reduce the antioxidant potential of the plants. Trichome development at this level appeared morphologically similar to the control plants, indicating that the glandular trichomes that release phenolics are developing properly and functioning normally within the leaf tissue.

Once a propagation system was established, plants were screened for potential medicinal value based on phenolic content and antioxidant potential. It is common that genetic variability and environmental factors influence the production of antioxidants in plants (Murch et al., 2004; Balasundram et al., 2006). This genetic variation is unfavourable for production of medicinal plants as standardized quantities of medicinal compounds need to be included on the labels (Government of Canada, Health Canada, 2013). Identification of antioxidant rich plant was determined through the DPPH antioxidant scavenging assay where an individual plant was selected and named “Vrinda” (Chapter 4). This plant was identified from a seed lot that contained other plants with high antioxidant potential in the leaf tissue, and was found to contain over
1400 µmolTE/g. This plant was further analyzed for growth characteristics to determine its commercial viability. Plants grown in outdoor conditions appeared darker with almost purple leaves, while greenhouse plants remained green. This difference may be due to the increased phenolic compound production including the pigment family anthocyanins to protect the plants from stress. Plants grown in different conditions were found to have no significant difference in antioxidant or phenolic potential, ideal for generating a consistent natural health product. Different tissues contained variable concentrations of phenolic compounds, with leaf tissue being relatively rich.

“Vrinda” was also analyzed for the antioxidant neurotransmitters melatonin, serotonin and GABA. These compounds are found in humans and influence circadian regulation, sleep, mental health, and have beneficial properties. Identification of the presence of melatonin and serotonin were found in the leaf tissue of in vitro, field and greenhouse grown plants for the first time in holy basil. No difference between field and greenhouse cultivated plants was observed, however, variation among individual plants was apparent; indicating the production of these compounds may not be regulated in the same manner as other antioxidants. These findings are new for holy basil, and may have implications for the medicinal potential of holy basil and to develop products with high concentrations of melatonin, serotonin, and other antioxidant compounds.

“Vrinda” was morphologically assessed from seed to maturity in greenhouse conditions and compared to an accession with lower antioxidant activity (Chapter 5). This was conducted to determine the growth characteristics for large scale production of this high quality plant. Increased germination rates of seeds collected from Vrinda were found, along with delayed flowering and visibly compact plants. These qualities are
beneficial from a production standpoint. The delayed flowering response is ideal in the case of holy basil as leaves are typically used in for medicinal preparation, and plant with an extended vegetative period can be harvested more frequently. The compact nature of the plant, with a less bushy appearance allows for large populations to be able to be grown in less area. All of these qualities indicate that seed derived from Vrinda would be suitable for commercial production through traditional cultivation practices. Successful collection of seed populations from cloned plants of “Vrinda” demonstrates the feasibility of an alternate system of propagation. This seed population may also be useful for further screening of phytochemical variations using mutagenic treatments.

Using media additives and hormones, plants were cloned in vitro while maintaining growth regulations put in place by Health Canada for NHPs. The plant “Vrinda”, was identified as being a high antioxidant medicinal plant, and was characterized morphologically and chemically. The discovery of high melatonin and serotonin levels in “Vrinda” open new doors for the medicinal usage of the plant. Using the developed protocol, large populations of plants with increased medicinal activity can be established which will assist in maintaining germplasm and uniformity of the plants as well as being able to explore the plant further in detail regarding the medicinal value.
References


AGRI-FOOD CANADA (2012). Introduction to Medicinal Plants - Agriculture and Agri-Food Canada (AAFC).


GOVERNMENT OF CANADA, (2013). Natural Health Products Regulations.


KUMARASWAMY, M. AND ANURADHA, M. (2010). Micropropagation of Pogostemon cablin Benth. through Direct Regeneration for Production of True to Type Plants. Plant Tissue Culture and Biotechnology 20, 81–89.


PHYTOTECHNOLOGY (2011). *Agars and Other Gelling Agents.* Kansas, USA.


WORLD HEALTH ORGANIZATION (2002). WHO Monographs on Selected Medicinal Plants, Volume 2. World Health Organization


