Induction of Autotetraploidy and Characterization of the Effects of Genome Duplication on Native Ornamental Species *Monarda punctata* and *Monarda fistulosa* (Lamiaceae)

By

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ABSTRACT

INDUCTION OF AUTOTETRAPLOIDY AND CHARACTERIZATION OF THE EFFECTS OF GENOME DUPLICATION ON NATIVE ORNAMENTAL SPECIES *MONARDA PUNCTATA* AND *MONARDA FISTULOSA* (LAMIACEAE)

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Lamina, petiole and nodes of *Monarda punctata* and *M. fistulosa* were tested to develop an *in vitro* propagation method using a range of seven BAP concentrations (0-25µM). Nodes produced significantly more shoots in both species and the optimal hormone concentration was 25µM. Unrooted plantlets were acclimatized using a rooting powder and directly placing them into a soil mixture. To induce autotetraploidy, oryzalin and trifluralin were added into the medium in a range of eight concentrations (0-120µM) and nodes were exposed for 1, 3 or 6 days. A total of 43 *M. punctata* and 124 *M. fistulosa* tetraploids were identified and significant correlations between stomatal length and ploidy level were found. Finally, in *M. punctata* TPC was increased with ploidy level while %AA was unaffected. *M. fistulosa* had a decrease in TPC and %AA with an increase in ploidy level. However, one autotetraploid had a significant increase in TPC and %AA.

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LIST OF ABBREVIATIONS

MS	Murashige and Skoog
PGRs	Plant Growth Regulators
IAA	Indole-3-acetic Acid
IBA	Indole-3-butyric Acid
BAP	6-benzylaminopurine
PPM	Plant Preservative Mixture
AC	Activated Charcoal
DMSO	Dimethyl sulfoxide
AA	Antioxidant Activity
TPC	Total Phenolic Content
DPPH	Diphenylpicrylhydrazyl
FCR	Folin-Ciocalteau reagent
GAE	Gallic Acid Equivalent

x

1.0 Chapter 1: Introduction and Review of *Monarda punctata* and *M. fistulosa*

1.1 General Introduction of *M. punctata* and *M. fistulosa*

Autopolyploidy is often observed in plants and can occur both naturally and synthetically. Polyploidy has been associated with changes in almost every level of growth and development including anatomical, structural and/or biochemical processes (Dhawan and Lavania, 1995). Particular interest in this phenomenon is focused on the ability of induced polyploidy to increase the overall visual appeal of a plant as well as the potential to increase plant tolerance to low input environments, essential oil and secondary metabolite production. The species to be studied can become model systems when examining the effects of genome duplication on all aspects of growth and development including morphology, anatomy, physiology, various stress responses and secondary metabolite production and accumulation.

The genus *Monarda* is part of the mint family (Lamiaceae) and consists of 15-17 species which are divided into 2 sub-genera (Scora, 1967a). The genus is widely distributed throughout North America ranging from Canadian prairies south through Texas and Mexico and east to the Atlantic Ocean, although its center of origin is thought to be in Northern Mexico and Texas (Scora, 1967a). *Monarda fistulosa* and *Monarda punctata* were selected because of their native status in Canada, as well as the known medicinal compounds that have been isolated from their essential oils (Johnson *et al.*, 1998; Scora, 1967b; Yamada *et al*., 2010; Zhilyakova, *et al.*, 2009). These two species were also selected as they have different chromosome numbers and are divided into the two different sub-genera of the *Monarda* genus.

M. punctata belongs to the sub-genus *Cheilyctis* section *Cheilyctis*, which is defined by an interrupted spike floral formation and a chromosome number of 2n=2x=22 (Scora, 1967a). *M.*

fistulosa is part of the *Monarda* sub-genera which is classified by its terminal flower morphology and chromosome number of 2n=2x=36 (Scora, 1967a). *M. punctata* is generally shorter in height and is between 0.15 and 0.91 m with a spread of 0.22 to 0.30 m (Anderson, 2003), compared to *M. fistulosa* which can reach a height of 0.61-1.20 m with a spread of 0.61-0.91 m (Anderson, 2000). *M. punctata* is commonly found in dry and sandy soils and it native to Ontario and Quebec and southward into New Mexico and Florida (Anderson, 2003). *M. fistulosa* is more broadly adapted with the unique ability to grow in adverse conditions. It is extremely widespread throughout North America and is native to all but three provinces, two territories and two states (Anderson, 2000). The terpenes, thymol and carvacrol have been recently isolated from the essential oils of young leaves of *M. punctata* (Scora, 1967b; Yamada *et al*., 2010) and *M. fistulosa* also has a high content of essential oils (2.4% dry weight) (Zhilyakova, *et al.,* 2009), rich with thymoquinone, thymol, and carvacerol (Johnson *et al.*, 1998; Scora, 1967b).

While neither of these species currently has a recorded *in vitro* propagation system, the intention is to develop a system which can be used to then induce tetraploidy. Chromosome duplication has been reported to have higher rates of success *in vitro* as well as a higher stability of ploidal level in the plants derived (Zhang *et al.,* 2008). Plant tissue culture or *in vitro* propagation is a system in which cells, tissues or organs are placed in an aseptic, controlled environment on artificial nutrient medium and induced to regenerate and produce new, complete plantlets. This type of system has several distinct advantages: the ability to reproduce large numbers of plants quickly in a relatively limited space, the process can occur at any time of the season, the resulting plantlets are generally genetically identical and free of disease (Jain and Ochatt, 2010). These are especially important traits for ornamentals where there is an industry standard to produce uniform plants with identical flower colours and morphology as well as the

ability to meet the requirements of consumers all year round (Jain and Ochatt, 2010). Also, the ability to reproduce large numbers of plants in a relatively short period of time is beneficial when the rate of polyploidization may be low.

1.2 *In Vitro* Propagation

In vitro propagation is a complex system that relies on an intricate relationship between several factors in order to successfully propagate new plantlets from somatic tissues. First the type of medium to be used, as well as any additional supplements, must be considered. Media are generally made by combining a mixture of inorganic salts with distilled water, supplements, vitamins, plant growth regulators and often a gelling agent. The most commonly used combination of inorganic salts is known as Murashige and Skoog (MS) media (Smith and Gould, 1989). The major components of MS media, which distinguishes its formula from other media mixtures, are the high amounts of nitrate, potassium and ammonium (Smith, 2012).

A source of carbohydrate must also be added to any salt mixture, as cells *in vitro* are generally less photosynthetically active and require supplemental sugars. Commonly, sucrose or glucose is added at concentrations between 2-5%, but other sources such as fructose or starch have also been used (Smith, 2012). Vitamins are also commonly added to media as additional supplements, as they have catalytic functions in enzymatic reactions, and B vitamins tend to be the most important for cell tissue culture (Smith, 2012). Another supplement which has many functions in cell and plant development is the hexitol, myo-inositol. Myo-insitol's known functions include: cyclitol biosynthesis, storage of polyhydric compounds as reserves, germination of seeds, sugar transport, mineral nutrition, carbohydrate metabolism, membrane

structure, cell wall formation, hormonal homeostasis, stress physiology and a growth enhancer *in vitro* (Smith, 2012).

Finally, some of the most important compounds required to induce de-differentiation, cell division and structure formation are known as plant hormones, or plant growth regulators (PGRs). There are several classes of PGRs, with auxins and cytokinins being the two most commonly used in plant tissue culture. Auxins are involved in cell division and root formation, however at high concentrations auxins have been known to inhibit morphogenesis (Miller, 1961). Naturally occurring auxins include indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), while 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) are synthetically derived (Smith, 2012). Cytokinins are used to promote cell division and shoot proliferation and morphogenesis (Miller, 1961). They include kinetin (Kn), 6-benzylaminopurine (BAP); zeatin (Zn) and N^6 (^{2}-isopentenyl)-adenine (2iP) (Miller, 1961). Some media can be used in liquid form; however, many media have the addition of a gelling agent to create a solid support. The most commonly used agent is washed and purified agar isolated from seaweed, while other chemicals such as Gelrite, which is a polysaccharide isolated from a fermentation process in a *Pseudomonas* species, can also be used (Kang *et al.,* 1982). The tissue source placed into *in vitro* culture is known as the explant. Some commonly used tissues for explants include: shoot tips, lateral bud, lamina, petiole, stem, cotyledon and hypocotyl (Smith, 2012).

While the examples listed above are the more commonly used tissue donors for explants, there has been successful propagation reported with a variety of tissue sources including: excised ovules, isolated pollen microspores and roots. The ability of a species to be propagated *in vitro* can be greatly limited by the tissue used to initiate cultures and examples of this have been

widely documented. For example, a study examining shoot meristems, internodes, nodes and petioles as explant donors in *Mentha piperita* documented large variability in the regeneration capacity between the four explant types (Sarwar *et al.,* 2009). On a ½ strength MS medium with 6.97 µM kinetin, nodal explants had a shoot regeneration frequency of 65% which was comparable to shoot meristem donors which had a frequency of 55%; however both internode and petiole explants failed to produce any plantlets on this medium (Sarwar *et al.,* 2009). The highest documented regeneration from internode and petiole explants was 50% and 40%, respectively, while shoot meristems and nodal sections both achieved a frequency of 85% when placed on different media (Sarwar el al., 2009).

Selecting the appropriate tissue is important not only for species preferences, but also for determining the method of regeneration for the explant during *in vitro* propagation (Litz, 1993; Sangwan *et al.,* 1997). Explants such as nodal sections and apical meristems most often undergo direct regeneration (Sangwan *et al.,* 1997). In this method of regeneration, a complete new plantlet is formed directly on/by the explant tissue because these explants possess pre-existing organized meristems, requiring only a cue to begin differentiating and forming a new plantlet (Sangwan *et al.,* 1997). Direct regeneration is quick, efficient and minimizes potential variation arising from extended periods in culture. Embryogenesis is another method of *in vitro* regeneration where cultures will first form small embryos which then develop into plantlets (Terzi and Loschiavo, 1990). This can occur either through direct methods (i.e. the explant tissue forms embryos directly on the tissue) or through indirect methods (i.e. the explant will first dedifferentiate into a callus state and then the formation of embryos will occur from the callus) (Terzi and Loschiavo, 1990; Sangwan *et al.,* 1997). Direct regeneration is preferred in for rapid propagation in order to obtain the greatest number of plantlets in the shortest amount of time

(Hicks, 1994). However, indirect regeneration and embryogenesis can be useful tools in other formats. Indirect regeneration has several biotechnological purposes and embryos recovered from embryogenesis can be encapsulated and used to produce synthetic seeds (Terzi and Loschiavo, 1990).

While there are currently no documented tissue culture protocols for *Monarda*, there have been many successful systems developed within the related Lamiaceae family. Some early work completed within the family on Sweet Basil (*Ocimum basilicum*) used a standard MS medium and added the supplementations sucrose (3%), myo-insitol (100mg/L) and agar (0.8%) (Sahoo *et al*., 1997). Using nodal sections containing a single axillary bud as their explant tissue, combinations of 0-8.9µM BAP and 0-2.4µM gibberellic acid (GA_3) were used to promote shoot regeneration. Within 10-12 days of explants being placed on MS medium containing 4.44µM of BAP, 85% demonstrated an initial bud break, developing a single shoot (Sahoo *et al*., 1997). The explants were then transferred to media containing 1.11µM of BAP and within 10-15 days the explants had formed multiple shoots, and by 30 days each explant had an average of 10.5 shoots that on average were 4.8cm long, had 4-6 leaves and 3-4 nodes (Sahoo *et al*., 1997). Once shoots reached a length of 3-4cm, plantlets were transferred to a ½ strength MS medium containing IAA (0-5.71µM), IBA (0-4.92µM) or NAA (0-5.37µM) for rooting. IBA was the most effective of the three auxins, and induced rooting in 93% of the explants at a concentration of 4.92µM (Sahoo *et al*., 1997). Plantlets were also successfully subcultured as nodal sections on MS medium with 1.11µM BAP at four week intervals, with a multiplication frequency of 100% during the first three subcultures (Sahoo *et al*., 1997).

Work done in another member of the Lamiaceae family, French Lavender (*Lavandula pedunculata*) used axillary buds and nodal sections as the explant sources which were then

cultured on a MS medium supplemented with sucrose (3%), ascorbic acid (10mg/L) and agar (0.6%) (Zuzarte *et al*., 2010). Zuzarte *et al*., (2010) tested only the PGR BAP in concentrations of 0-2.22µM and found that for both tissue sources, a concentration of 1.11µM BAP produced the highest amount of shoots; however, shoot development was more rapid from nodal explants than axillary buds. After shoot development, the plantlets were placed onto a hormone free basal MS medium to encourage shoot elongation and discovered roots regenerated spontaneously in these conditions (Zuzarte *et al.,* 2010). The essential oil compositions of *in vitro* grown plantlets and field grown plants were also examined and it was found that the compositions only had very minor deviations in the secondary constituents of the oils (Zuzarte *et al.,* 2010). Other tissue culture methods developed within the Lamiaceae family include: Hyssop (*Hyssopus officinalis*) (Toma *et al.*, 2004); Field Mint (*Mentha arvensis*) (Maity *et al*., 2011); and Indian Coleus (*Coleus forskohlii*) (Dube *et al.*, 2010) with optimal hormone combinations of: 4.92µM IBA, 4.44µM BAP and 9.29µM Kn + 5.71µM IAA respectively.

1.3 Induced Polyploidy

Induced polyploidy is typically carried out *in vitro* through the use of an antimitotic agent. There are several chemicals that can be used, with the three most common being colchicine, oryzalin and trifluralin. Colchicine is a naturally derived compound and is prescribed for the treatment of acute flare ups of gout and familial Mediterranean fever (FMF) in humans (FDA, 2009). It works to induce polyploidy in plants through the disruption of spindle fibre formation, preventing nuclear and cellular division (Ascough *et al.*, 2008). Oryzalin and trifluralin were developed synthetically as herbicides for weed control and act to disrupt microtubule assembly during cell division (Ascough et al., 2008). Although the effectiveness of these compounds is highly dependent upon concentration, duration of exposure and explant type,

it is often thought that oryzalin and trifluralin are more effective at inducing polyploidy in plants as they have a higher affinity for plant tubulins than colchicine does (Ascough *et al*., 2008; Kermani *et al.*, 2002). Other work has also found that these two synthetic compounds have a higher percentage of stable polyploidy production and a higher survival rate at lower concentrations than colchicine (Ascough *et al*., 2008). Previous work optimizing chromosome doubling procedures in ornamental species have successfully induced polyploidy with oryzalin, colchicine and trifluralin, in both solid and liquid media systems and with a variety of explant tissues, including unrooted plantlets, excised shoot tips, cotyledons and nodal sections. The concentration of antimitotic agent used to induce polyploidy has been inconsistent in previous literature, ranging from 100-37,500 µM of colchicine, 0.5-1500 µM of oryzalin and 1-24 µM of trifluralin (Cohen and Yao, 1996; Dhooghe *et al.*, 2009; Kermani *et al*., 2002; Khosravi *et al.*, 2007; Stanys *et al.*, 2005; Thao *et al.*, 2003; Vainola, 2000; Zhang *et al.*, 2008).

A study conducted on the *in vitro* induction of tetraploidy in Ranunculus (*Rancunculus asiaticus*) examined the efficiency of the three anti-mitotic inhibitors colchicine, oryzalin and trifluralin on unrooted plantlets (Dhooghe *et al*., 2009). The optimum dosage and duration of exposure were found to be significantly different between the three chemicals, with colchicine producing 23.3% tetraploids at a concentration of 200µM when applied for 16 or 24h while oryzalin and trifluralin both produced the most tetraploids when plantlets were exposed for 10 weeks at concentrations of 1µM (18.5%) and 2µM (27.5%) respectfully (Dhooghe *et al*., 2009). Another study conducted by Stanys *et al.* (2005) on inducing polyploidy in Japanese quince (*Chaenomeles japonica*) tested oryzalin and colchicine on microshoots and cotyledon explants *in vitro*. Oryzalin resulted in the highest number of tetraploids in microshoots at 30 or 40 µM for 1-2 days and in cotyledons between 20 and 40 µM for 1 day, while colchicine was found to be

most effective at concentrations of 15,000 µM and 22,500 µM (Stanys *et al.*, 2005). Oryzalin was also used to induce polyploidy in another member of the Japanese quince family, *Rosa* spp. and produced very different results (Kermani *et al*., 2002). Kermani *et al*. (2002) found that oryzalin induced the highest frequency of polyploids in shoot tips of *Rosa* when applied at a concentration of 5 µM for 14 days or in thin nodal sections when 5 µM was applied for 1 day. Finally, a study carried out by Khosravi *et al*. (2007) looked at the effect of inducing tetraploids in three *Rosa* cultivars, *Rosa hybrida* cv. Iceberg and Akito and *R. persica* and found that when nodal explants were treated with 6µM of oryzalin for 24h 6.3%, 0% and 60% were polyploid, respectively (Khosravi *et al*., 2007).

Chimeras, or mixoploids, are another frequent result when using antimitotic agents to induce polyploidy. Mixoploids are a single plant which has both 2x and 4x cells and can frequently revert back to diploidy after a few months. These plants can make the usage of phenotypic traits for identification of genome duplication events unreliable (Ascough *et al.*, 2008). Several studies have suggested that the percentage of mixoploids produced is related to the concentration and duration used, with longer durations and higher concentrations reducing the incidence of chimeras (Ascough *et al.*, 2008; Thao *et al*., 2003; Zhang *et al*., 2008). Most often, when a mixoploid is identified, its ploidy level is reconfirmed after several weeks to check for reversion, or the plant is simply discarded. Thao *et al*. (2003) identified mixoploids in *Alocasia* and found that the appearance of chimera's leaves was largely variable and the leaves were often deformed or asymmetric. The authors believed that these deformities were due to the presence of both tetraploid and diploid cells constituting a single tissue.

1.4 Effects of Polyploidy on Morphology and Anatomy

There have been several studies conducted to test the effect of induced and natural polyploidy on morphological traits in ornamental plants. One recent study examined the effect of 2x and 4x cytotypes of *Spathiphyllum wallisii* (peace lily) on the plant's morphology and growth (Van Laere *et al*., 2011).Significant differences among organ formation, flower development and growth between tetraploid and diploid progenitors was reported. It was also found that tetraploid *S. wallisii* had fewer leaves per stem but the leaves were thicker and more ovate, with a decreased length/width ratio (Van Laere *et al*., 2011). Finally a shorter and thicker flower stalk was noted, and the tetraploid plants were reported to have produced fewer shoots and total biomass than the diploid plants (Van Laere *et al*., 2011).

Several other studies have documented morphological differences observed between the two cytotypes, and with the exception of overall plant size, most studies agree on a general trend of phenotypic changes associated with increased polyploidy. Leaves of polyploids are thicker, larger and a darker green colour and they tend to experience a decrease in the leaf length/width ratio, as well as an altered leaf shape and an overall decrease in total leaf number (Allum *et al*., 2007; Emsweller and Brierly, 1940; Emsweller and Ruttle, 1941; Gu *et al*., 2005; Kermani *et al.*, 2002; Kobayashi *et al*., 2008; Van Laere *et al.*, 2011; Levin, 1940; Rose *et al*., 2000). Other leaf changes associated with polyploidy that have been observed include a more crinkled appearance in *Buddleja globosa* (Rose *et al.*, 2000), an increase in leaf angle in *S. wallisii* (Van Laere *et al*., 2011) and an increase in leaf weight in *Rosa rogosa* (Allum *et al.*, 2007). Morphological changes associated with flowering include an increased flower diameter, delayed and possibly increased time to flowering and length of flowering, an increase in petal number, more intense and new coloration and/or shorter, thicker floral spikes (Allum *et al*., 2007; Emsweller and Brierly, 1940;

Emsweller and Ruttle, 1941; Gu *et al*., 2005; Kermani *et al.,* 2002; Kobayashi *et al*., 2008; Van Laere *et al.,* 2011; Levin, 1940; Oates *et al*., 2012; Rose *et al*., 2000). Finally, changes in height of the tetraploid plant are generally observed, however whether the change will result in a taller, or shorter plant is controversial. Tetraploidy in *Rosa* (Kermani *et al*., 2002), *Rudebekia* (Oates *et al*., 2012), *Petunia* (Levin, 1940) and *Salvia* (Kobayashi *et al*., 2008) has resulted in taller plants with longer internodes than their diploid progenitors. In contrast, tetraploidy in *Lilium* (Emsweller and Brierly, 1940), *B. globosa* (Rose *et al*., 2000), *S. wallisii* (Van Laere *et al*., 2011), and *Zizyphus jujubz* (Gu *et al.,* 2005) have resulted in shorter plants, with shorter internodes, reduced biomass and a more compact growth habit.

There are several anatomical changes associated, or thought to be associated, with the physical changes observed with an increase in ploidy levels. First, with an increase in nuclear content, tetraploids see an increase in cell volume to approximately twice the size of a diploid; however, the cell surface area only undergoes an average increase of 1.5 times its original size (Emsweller and Ruttle, 1941; Lavania, 2012). The overall increase in cell volume and surface area may contribute to the increased size and girth of polyploid organs, such as leaves, flowers and increased internode lengths (Lavania, 2012). An increase in the thickness of the upper and lower epidermis also occurs and may contribute to an increased leaf thickness (Li *et al*., 1996). However, the decrease in the cell volume/surface area ratio is thought to cause a reduction of growth rate in polyploidy cells, a reduced rate of enzymatic processes as well as altering the percentage of chromatin in contact with the nuclear membrane (Emsweller and Ruttle, 1941). This slowed rate of development is often associated with a later flowering time (Lavania, 2012), while the decreased enzymatic reactions could account for the decreased body size observed in some induced tetraploids and the increased contact between chromatin and condensed regions of

the nuclear membrane has been suggested to alter gene expression and regulation (Emsweller and Ruttle, 1941). Finally, Warner and Edwards (1989), examined the amount of chlorophyll per cell and photosynthetic rates among several cytotypes of *Atriplex confertifolia,* and found a positive linear correlation of $r^2 = 0.957$ and 0.965, respectively, between these two characters and ploidy level. This increased chlorophyll accumulation per bundle sheath cell in polyploids is likely the cause of the darker green leaf phenotype.

1.5 Effects of Polyploidy on Physiology

Polyploidy has also had implications in increasing the plant's ability to adapt to drought conditions, temperature stresses and disease (Levin, 1983). A study conducted by Li *et al*. (1996) on *Betula papyrifera* found that the 4x cytotype had a lower osmotic potential at saturation allowing them to maintain higher turgor in low water compared to their diploid progenitors. It was also noted that tetraploids had a higher net photosynthetic rate, rate of CO_2 assimilation per unit leaf area and a higher stomatal conductance at the same level of drought stress as diploid cultivars (Li *et al*., 1996). Higher photosynthetic rates in polyploids under drought stress have also been found in *Lonicera japonica* (Li *et al.,* 2009) and *Atriplex confertifolia* (Warner and Edwards, 1989). Another study examining the effect of drought on diploid and tetraploid *Spathiphyllum wallisii* found that the tetraploid plants were much more tolerant to drought conditions than their diploid counterparts (Van Laere *et al.*, 2011). The tetraploids in general had a smaller stomatal density but larger pores and a lower stomatal resistance. After 15 days of increasing drought stress, the diploid experienced a significant decrease in both relative water content and leaf water potential while the tetraploids showed either no decrease, or only a slight decrease in both of these measurements (Van Laere *et al.*, 2011). Induced polyploidy has also yielded a novel resistance to a form of snapdragon rust (Emsweller and Ruttle, 1941), increased

resistance to *Sclerotinia* clover rot in *Trifolium pretense* L. (Arseniuk, 1989) and improved heat tolerance in *Dioscorea zingiberensis* (Zhang *et al.,* 2010).

There are several reasons why polyploids tend to surpass their diploid counterparts under various stress conditions. First, they are thought to be more drought tolerant because of their ability to maintain photosynthesis under high levels of stress, when diploids would normally cease photosynthesis (Li *et al.,* 1996). This is related to the increased degree of stomatal opening under drought as compared to the diploid, allowing polyploids to continue photosynthesizing while maintaining leaf water content and turgor pressure (Li *et al*., 2009; Van Leare *et al*., 2011). A few anatomical adaptions have also been cited as causes for their increased drought tolerance. These include a thicker upper and lower epidermis which allows for lower water evaporation from the leaf (Li *et al.,* 2009) and a decreased stomatal density commonly observed, which is thought to contribute to the decreased water loss in tetraploids (Tal and Gardi, 1976). An ability of polyploids to better adapt to heat stress is speculated to have been caused by the expression of a duplicate gene (Zhang *et al.,* 2010), while their increased pest and pathogen resistance is likely the result of high production of secondary metabolites, such as alkaloids and terpenes (Levin, 1983).

1.6 Effects of Polyploidy on Phytochemistry

The study conducted by Zhang *et al.,* (2010) on the heat tolerance of tetraploid *D. zingiberensis* examined the antioxidant activities of the two cytotypes while under heat stress. They found that after being placed in a room at 42°C for 24 hours, diploid plants experienced a significant drop in the rate of antioxidase activity, while the tetraploid showed only a minor decline (Zhang *et al*., 2010). This demonstrates that polyploids potentially have a higher affinity

to continue enzymatic reactions under various environmental conditions than their diploid progenitors. It has been previously shown that induced polyploidy can result in an increased concentration of essential oils as well as a greater diversity of secondary metabolites (Dhawan and Lavania, 1995). Studies conducted by Janaki-Ammal and Gutpa (1996) examined naturally occurring cytotypes of lemon grass (*Cymbopogon flexuosus*) and found that there was a positive correlation between chromosome number and essential oil concentration. Other work completed in the autotetraploid *Briza media* found that the essential oil constituents differed between cytotypes, and instances in which secondary metabolites were present in the tetraploid progeny but absent in the diploid progenitor was reported (Murray and Williams, 1976).

Lavania *et al.,* (2012) studied induced autotetraploids of eight species of *Cambopogon sprengel* and the effects of genome duplication on their essential oil production. They found that increased genome duplication resulted in an increase of essential oil concentration and a direct relationship between oil constituents and the effect of genome duplication on plant mass was cited (Lavania *et al.,* 2012). These results suggested that species whose oil is alcohol rich will experience an increased plant mass, while plants with aldehyde rich oils tend to show a decrease in overall size after genome duplication (Lavania *et al.,* 2012). The autotetraploid *Cambopogon sprengel* had an increased size of essential oil secreting cells, which could have caused the increase in oil volume however, the number of these specialized cells was less than in the corresponding diploids (Lavania *et al.,* 2012). They also speculated that the relationship between oil content and body size was an effect of the metabolic expense related to the constituent synthesis (Lavania *et al.,* 2012). The metabolic pathway used for terpenoid synthesis is not shared with other pathways in the cell making it more of a resource drain on the plant, and in a newly synthesized autotetraploid, this becomes even more of an issue because the cells may now

possess the enzymatic capacity to produce terpenoids at twice the capacity (Lavania *et al.*, 2012). Finally, the instances of altered essential oil profiles are thought to have been caused by a functional de-repression of a previously silent gene (Dhawan and Lavania, 1995).

The ability of a polyploid to continue enzymatic reactions while under heat stress at a rate comparable to its normal state emphasizes the potential for tetraploid plants to produce larger volumes of essential oils and antioxidant metabolites under any condition. The changes in essential oil production and enzymatic reactions are thought to be related to the whole genome duplication that autopolyploids undergo resulting in larger cell volumes and multiple alleles per locus (Lavania *et al*., 2012; Dhawan and Lavania, 1995). Zhang *et al*., (2010) hypothesized that the changes observed were caused by the expression of a duplicate gene.

1.7 Experimental Overview

An *in vitro* propagation method for both *M. fistulosa* and *M. punctata* was first developed to allow for the induction of chromosome doubling and ultimately the completion of a phytochemical study to determine the effects of autopolyploidy. Once a tissue culture system had been established, the concentration and duration of exposure to oryzalin or trifluralin required to induce polyploidy in *M. punctata* and *M. fistulosa* were tested and optimized. Polyploid induction was tested using flow cytometry to identify tetraploid individuals. Increased stomatal sizes were also found to be an indicator of ploidy and aided in the selection of plantlets. Once these plants were successfully identified the final effect of genome duplication on the total antioxidant and total phenolic content of leaf extracts from genotypically pair tetraploid and diploid cytotypes was determined. These two assays are used as an indicator of potential changes which may occur in the essential oils as a result of induced tetraploidy.

1.8 Objectives and Hypotheses

This research has three objectives and associated hypotheses:

1. To develop a functional *in vitro* propagation method for *M. punctata* and *M. fistulosa*.
    a. *Monarda punctata* and *Monarda fistulosa*, like other members of the Lamiaceae family, can be successfully propagated *in vitro.*
2. To develop and optimize protocols to induce polyploidy for *M. punctata* and *M. fistulosa* using *in vitro* plantlets and to successfully verify autotetraploids through cytological studies.
    a. The concentration and duration of exposure to oryzalin or trifluralin required to induce polyploidy in *M. punctata* and *M. fistulosa* will vary, with some combinations proving to be lethal while others may result in little to no polyploidization.
    b. There will be morphological traits which will aid in the identification of polyploid plants (e.g. increased stomatal sizes).
3. To assess and quantify phytochemical differences of the leaf extracts of diploid and autotetraploid cytotypes.
    a. An increase in ploidy will result in an increased production of phenolic compounds and antioxidant activity compared to the diploid progenitors.

2.0 Chapter 2: Development of an *in vitro* Propagation Method for *M. punctata* and *M. fistulosa*

Abstract:

A method to propagate two species of *Monarda in vitro* was successfully developed using a heterogeneous population of greenhouse grown explant donors for induction. Leaf and petiole sections were determined to be unsuitable explants for both *M. punctata* and *M. fistulosa* in the culture conditions tested. Nodal sections however, were able to regenerate plantlets *in vitro* on solid MS media at almost all concentrations of BAP tested, as well as with no additional hormone supplementation. The highest rate of regeneration observed was obtained when nodal sections were placed on medium containing 25µM of BAP. Regeneration was found to be significantly affected by the specific plant genotype. Plantlets were successfully acclimatized over a two week period without an additional *in vitro* rooting step. These results demonstrate the possibility of large scale *in vitro* propagation and a simple method of rooting for both *M. punctata* and *M. fistulosa*. They also illustrate how genetic variability can largely impact the culturability of a species *in vitro* and how this can affect propagation studies with native populations which are heterogeneous by nature.

2.1 Introduction:

The genus *Monarda* is comprised of 16 species of flowering annuals, biennials and perennials (Scora, 1967a). The species of this genus are considered to be native across much of North America, and the genus is subdivided into two sub-genera, *Monarda* and *Cheilyctis* (Scora, 1967a). The genus *Monarda* belongs to the family Lamiaceae and like other members of the family are regarded for their flowers, essential oils and fragrance. Two species of particular

interest are *Monarda punctata* and *Monarda fistulosa*. Both species are native to Ontario, contain medicinal compounds in their essential oils, and produce large showy flowers (Johnson *et al.*, 1998; Scora, 1967b; Yamada *et al*., 2010; Zhilyakova, *et al.*, 2009).

M. punctata, also known as Horsemint or Spotted Bee Balm, belongs to the sub-genus *Cheilyctis* and has a chromosome number of 2n=2x=22 (Scora, 1967a). Varieties of this species can grow up to 100cm tall and produce an interrupted floral spike with white to pink bracts (Anderson, 2003; Scora, 1967a). Within each flowering whorl, multiple corollas form and have a defined upper lip which can be white to yellow in colour and are often spotted pink or purple (Scora, 1967a). Its leaves tend to be lanceolate or oblong and the number of trichomes can vary significantly (Scora, 1967a).

M. fistulosa has a chromosome number of 2n=2x=36 and belongs to the sub-genera *Monarda* (Scora, 1967a). This species is commonly known as Wild Bergamot or Bee Balm. It has a square stem and its leaves tend to be ovate or lanceolate with serrated margins (Scora, 1967a). It can reach up to 120cm in height and produces a terminal glomerulus flower which is purple in colour (Anderson, 2000; Scora, 1967a). Breeding research has been conducted within this species, and crosses have been made with its relative, *M. didyma* (Collicutt and Davidson, 1999).

One of the main objectives of the research program is to create tetraploid cytotypes of *M. punctata* and *M. fistulosa* in order to investigate the effects of chromosome number on essential oil production, antioxidant potential and overall ornamental characteristics. However, for the purposes of this study, an *in vitro* propagation method was first developed. *In vitro* propagation, or tissue culture, is a method which allows for the cloning and multiplication of a single plant

using a small piece of tissue, or an explant. By creating a medium which has the correct combination of micro- and macro- nutrients, plant growth regulators (PGRs) and inorganic salts, a small piece of plant can be manipulated to undergo de-differentiation and subsequent regeneration of complete new plantlets, organs or embryos. The first obstacle to this technique however, is selecting the correct medium components as well as the most regenerative explant tissue.

Various PGRs can be used in numerous combinations and concentrations to induce plant regeneration. The two most commonly used classes of PGRs are auxins and cytokinins (Gasper *et al.,* 1996). Auxins are involved in the initiation of cell division, cell expansion and meristem organization as well as helping to promote root formation in whole plantlets (Gasper *et al.,* 1996; Miller, 1961). Auxins include both naturally occurring compounds [indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA)] and synthetically derived compounds [1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D)] (Smith, 2012). Cytokinins are a class of PGR used to promote cell division, shoot formation and proliferation as well as aiding in the release of bud dormancy (Gasper *et al.,* 1996; Miller, 1961). Commonly used cytokinins include kinetin (Kn) and 6-benzylaminopurine (BAP) (Gasper *et al.,* 1996; Miller, 1961). A specific ratio of cytokinin and auxin is required in order to successfully promote the organized development of new plantlets and plant tissues (Gasper *et al.,* 1996).

This ratio is also dependent on the type of regeneration desired for the plant of interest. Different concentrations and combinations of PGRs can greatly affect the explant's behavior *in vitro* (Gasper *et al*., 1996) and the most common regeneration methods included organogenesis and somatic embryogenesis (Litz, 1993). Both of these pathways generally involve the re-programming of single somatic cells *in vitro* (Terzi and Loschiavo, 1990). Somatic

embryogenesis produces a bipolar embryo which typically has no vascular connection to the explant tissue or callus, while organogenesis produces a multicellular organ or structure which has a vascular connection with either the explant or the callus cells (Terzi and Loschiavo, 1990). Embryogenesis results in a new reproductive embryo structure which can be sub-cultured to produce a new plantlet or be encased in a gel substance to create a synthetic seed that can be planted directly in soil (Terzi and Loschiavo, 1990). Organogenesis gives rise to entirely new plantlets without the initial formation of embryos and therefore can produce large populations of plantlets quickly without the need to sub-culture embryos in order to obtain plantlets (Hicks, 1994). However, plantlets derived from organogenesis need to produce roots and be carefully acclimatized to non-sterile environments and soil mediums or continually sub-cultured for long term *in vitro* storage.

The selection of plant tissue, or explant, used for *in vitro* propagation can also affect the regeneration method and efficiency of the system (Litz, 1993; Sangwan *et al.,* 1997). A wide variety of explants have been used in various systems and include: shoot tips, leaves, petioles, nodal sections, cotyledons and hypocotyls (Smith, 2012). The response of various explants to *in vitro* propagation varies between species and the maturity of the selected explant tissue can also play a large role in the regenerative capacity of the species (Sangwan *et al.,* 1997).

The use of tissue culture to induce chromosome doubling is the preferred method for several reasons. First, literature suggests that application of chemicals to induce chromosome doubling through tissue culture results in a greater number of polyploids which have a more stable ploidy level than plants exposed to these chemicals by other methods (e.g. apical application, seed soak or floral dips) (Zhang *et al.,* 2008). Second, tissue culture is a quick and efficient method to produce multiple genetic clones of a single plant (Jain and Ochatt, 2010).

When plants respond poorly to chromosome doubling and the rate of polyploidization is low, *in vitro* propagation can allow for a larger population of polyploid clones to be obtained in a shorter period of time compared to seed propagation. A tissue culture method is also appealing as it allows for the maintenance and production of large populations of plants in a relatively small amount of space (Jain and Ochatt, 2010). Although there is interest in these two *Monarda* species for horticulture and medicine, there are currently no reports of tissue culture systems in either species.

For *in vitro* propagation of related members of the Lamiaceae family, nodal sections were the most commonly used tissue (Maity *et al*., 2011; Sahoo *et al*., 1997; Toma *et al.*, 2004; Zuzarte *et al*., 2010), but there are also reports of cotyledon and leaf explants used in sweet basil (*Ocimum basilicum*) (Dode *et al.*, 2003; Kintizios *et al.*, 2004). All studies examined used MS (Miller and Skoog, 1962) basal salts, 3% sucrose, and 100mg/L myo-insotol (Maity *et al*., 2011; Sahoo *et al*., 1997; Toma *et al*., 2004; Zuzarte *et al*., 2010). The concentration of PGRs varied, but the majority of plants examined achieved optimal regeneration using a combination of the cytokinin BAP and the auxin NAA or IBA (Dode *et al.*, 2003; Kintizios *et al.*, 2004; Maity *et al.*, 2011). However, it was noted that the use of an auxin was not always required in the initial media in order to promote plantlet formation (Fracaro and Echeverrigaray, 2000; Zuzarte *et al.*, 2010). Leaf explants of sweet basal and Coleus (*Coleus forskohlii*) responded well to kinetin as their cytokinin (Dube *et al.*, 2010; Kintizios *et al.*, 2004).

The objective of this study is to develop an efficient *in vitro* propagation procedure for regeneration of *M. punctata* and *M. fistulosa* as a system to manipulate ploidy levels and microrpropagate elite accessions. Leaf, petiole and nodal tissues were evaluated as explant donors for both plant species and a total of seven BAP concentrations were applied to each

explant tissue for a total of twelve weeks. It was hypothesized that moderate concentrations of BAP included in the media would induce regeneration through organogenesis with the highest efficiency.

2.2 Materials and Methods:

2.2.1 Explant Source

Seeds for *Monarda punctata* and *M. fistulosa* were obtained from Wild Flower Farms (Coldwater, Ont., Can.), and grown in the Edmund C. Bovey greenhouses under standard conditions at 24°C ± 2°C with 14 hours light and 10 hours dark (University of Guelph, Guelph, Ont., Can.).

2.2.2 Lamina and Petiole Culture

On the day of culture, petioles of healthy, undamaged leaves were cut from the stems of randomly selected plants in a vegetative growth stage. The lamina were then detached from the petiole and both the lamina and petiole were placed in beakers on ice until sterilization. The tissue was sterilized by placing it into metal tea strainers and submersing the strainer in 70% ethanol for 60 seconds, 15% bleach + 1% tween20 for 8 minutes and then rinsed three times for 3 minutes in sterile deionized water.

2.2.3 Nodal Culture

Sections of stem were cut from both species in the early flowering stages and the terminal bud as well as the leaves and petioles were removed. The sections of stems were selected randomly each day from a large population of donor plants with the only requirement being that the stems and plants appeared healthy. The sections were then cut into approximately

5 cm pieces and placed in beakers on ice until sterilization that day. They were rinsed for 15 minutes under cold running water to remove any dirt or debris. The explants were sterilized by placing them directly into a beaker containing 70% ethanol for 90 seconds, followed by a second bath of 15% bleach + 1% Tween 20 (Polyethylene glycol sorbitan monolaurate) (Sigma Aldrich, Oakville, Ontario) for 10 minutes, and were then rinsed in sterile deionized water in a series of three beakers for 3 minutes each. All beakers used for sterilization were placed on a stir plate at a low rpm to ensure all material was submersed in the sterilizing agents.

2.2.4 Media and Culture Conditions

All explants were placed onto a modified Murashige and Skoog (1962) medium (MS) with 3% sucrose, an MS vitamin mixture (100mg/L myo-Insitol, 2mg/L glycine, 0.5mg/L nicotinic acid, 0.5mg/L pyridoxine·HCl and 0.1mg/L thiamine·HCl) (Phytotechnology, Kansas, USA) and the media were supplemented with 0, 1, 5, 10, 15, 20 or 25µM of 6-benzylaminopurine (BAP) (Sigma Alrich, Oakville, Ontario). Due to high amounts of contamination from nodal sections, the media used for nodal culture were also supplemented with 2mL/L of plant preservative mixture (PPM) (Plant Cell Technology, Washington, USA). The media were then brought to a pH of 5.8 and 0.7% lab grade agar (Fisher Scientific, Canada) was added. Once autoclaved at 104kPa and 121°C for 20 minutes, approximately 25mL of media were poured into 100x15 mm petri dishes for leaf and petiole culture, or 100x25mm petri dishes for nodal culture and allowed to cool and solidify.

2.2.5 Culturing Explants

Leaves were cut into approximately 1cm x 1cm squares, making cuts along all four sides surrounding the midrib. The squares were placed abaxial side onto the surface of the medium and

all sides were gently pressed down to ensure maximal contact. Petiole and nodal explants were cut into 1 cm segments ensuring both ends of the petiole and node as well as any remain petiole on the nodal explants were cut. The sections were then placed length wise onto the medium surface and gently pressed down so approximately half of the width was submersed into the medium.

2.2.6 Experimental Design

All experiments were conducted as a randomized complete block design, consisting of five blocks with each block consisting of three replications of every BAP concentration in both species. A single block was completed in one day and each of the five blocks was harvested and plated on five different days. Each plate consisted of four pieces of tissue which were randomly selected from sterilized material. Cultures were maintained in a growth cabinet at 25±1°C, and illuminated with cool white fluorescent tubes. Leaf and petiole cultures were maintained under a 16 h photoperiod, while the nodal sections received 24h illumination. All cultures were sub-cultured to fresh media every 4 weeks.

2.2.7 BAP Evaluation for Nodal Sections

At each sub-culture all explants were measured and recorded. After twelve weeks, four parameters were used to identify the optimal regeneration medium. The methods used to evaluate the optimal concentration of BAP for nodal regeneration in both species were: 1) the average number of shoots produced per explant; 2) the percentage of nodal sections which gave rise to at least one plantlet; 3) the average number of shoots produced only by nodal sections calculated using the second parameter; and 4) the total number of shoots regenerated. The first parameter was obtained by dividing the total number of shoots produced by a given treatment over twelve

weeks by the total number of explants left uncontaminated at the end of the same period ([1] $\frac{\text{total \# of shoots}}{\text{\# of uncontaminated nodes}} = \text{average \# of shoots/node}$). The percentage of living nodal sections was defined as the percentage of explants which were able to regenerate at least a single plantlet in twelve weeks ([2] $\left[\frac{\text{\# of nodes that produced plantlets}}{\text{\# of uncontaminated nodes}}\right] x\ 100 = \%\ \text{alive}$). The average number of shoots produced by living nodal sections was calculated with information from the first two measurements ([3] $\frac{\text{total \# of shoots}}{\text{\# of nodes that produced plantlets}} = \text{average \# of shoots/alive node}$). The fourth method was calculated by multiplying the percentage of regenerative nodal sections by the total number of initial explants in order to obtain the total number of explants which would have been produced if contamination was negligible. This was then multiplied by the average number of shoots produced by the regenerative nodes ([4] [($\frac{\%\ \text{alive}}{100}$) * original # of nodes plated]*average shoots/alive node = total # of shoots/ originally plated nodes).

2.2.8 Preliminary Genotype and Positional Nodal Trial

In order to investigate the effect of genotype and nodal position/age on the regeneration efficiency of nodal explants, 12 *in vitro* plantlets from each species were selected ensuring that their parental donors were not related. Once 12 non-related individual plantlets were selected, they were removed from a basal MS medium and the uppermost and, bottom most nodes were excised, leaves were removed and the nodes were placed onto a petri dish containing 25µM BAP along with the two middle nodes (top-middle and bottom-middle). Each petri plate contained four nodes and represented one unreplicated individual. Plantlets were subcultured onto fresh medium every four weeks for a total of 12 weeks. Number of shoots produced, and length and node number of the shoots were recorded.

2.2.9 Rooting

On the day of transfer, any plantlets greater than 1.5 cm resulting from the cultures were removed from the explant before transferring to fresh medium, measured and randomly placed into a glass culture tube (25x150mm) containing 10 mL of a rooting medium. Rooting treatments consisted of a full strength or half-strength MS media, with or without 0.6% activated charcoal and were supplemented with 0 or 5µM indole-3-acetic acid (IAA) or 0, 2.5, 5, 7.5 or 10µM indole-3-butyric acid (IBA). All media were comprised of MS salts and vitamins and 3% sucrose, adjusted to a pH of 5.8 and autoclaved at 104kPa and 121°C for 20 minutes with 0.7% agar. Plantlets were maintained under the same growth cabinet conditions as the initial shoot regeneration cultures (25±1°C and 16 hour photoperiod).

2.2.10 Evaluation

After 12 weeks in culture, all remaining plantlets were measured and recorded, then discarded. Shoot regeneration was evaluated based on the average number of shoots per explant per plate, the percentage of explants producing shoots, and the average number of shoots per regenerating explant. Rooting was evaluated after 6 weeks and the following characters were measured: change in height and node numbers as well as the number of roots and the length of the longest root.

2.2.11 Acclimatization

Due to the lack of roots produced *in vitro* direct rooting methods were examined. Plantlets of both species were placed into culture tubes containing a basal MS media with no PGR or antibiotic additive and placed into a growth chamber (25±1°C and 16 hour photoperiod) to promote elongation. Once plantlets were over 15cm in height, they were gently removed from

the tube and medium was washed away. The height, node number, root number and root length were recorded and the basal end of the plantlet and any roots which were formed were dipped into StimRoot #1 powder. These plantlets were then placed into a 50 cell tray containing a 50:50 turface: Sunshine #4 (v/v) mixture ensuring the StimRoot portion was completely covered with potting mix. Trays were placed on a misting bed for seven days, and then placed on a bench lined with a moisture pad and covered with a plastic dome for another seven days. Survival was assessed after six weeks and before transplanting to 8.9 cm pots filled with the same soil mixture.

2.2.12 Statistics

The average number of shoots per explant; the percentage of explants producing shoots; the average number of shoots per regenerating explant and the total number of shoots produced by twelve nodal explants were analyzed using Proc Mixed in SAS 9.3 (SAS Institute, Inc., Cary, NC). For the following characteristics an arc-sin transformation was required to normalize the data and the values were then back transformed for reporting: average number of shoots per explant; the percentage of explants producing shoots and the average number of shoots per regenerating explant. When calculating the average number of shoots per explant, any explant within a treatment group which became contaminated before twelve weeks was concluded, was removed from the data and the average was calculated by divided by the total number of remaining explant tissues. Significance levels were set at $\alpha=0.05$ and means comparisons were performed using a Tukey's pairwise comparison. Linear regressions were tested for all parameters using Proc REG and model significance was set at $\alpha=0.05$. The total number of shoots produced from individual genotypes was analyzed using Proc GLM and each nodal position (i.e. top, bottom) was considered a block for the purpose of analysis with a total of four replicates. The effect of position was also analyzed using Proc GLM, with each genotype being

considered a replicate, totaling 12 blocks. The means from both the positional and genotype trials were compared using a Tukey's pairwise comparison (α=0.05). Data from the rooting experiments were analyzed using Proc GLM and a means comparison using a Tukey's pairwise comparison was completed, which is consistent with a completely random design.

2.3 Results:

2.3.1 Contamination from *in vivo* Grown Plant Material

Three tissues were used for both species to initiate *in vitro* cultures from greenhouse grown plants. The sterilization protocol proved to be effective for laminae and petioles, with *Monarda punctata* and *M. fistulosa* having contamination rates of 20% and 14% for lamina tissues, and 28% and 25% for petiole explants after 90 days, respectively (data not shown). However, nodal sections had a much higher rate of contamination and due to this the experiment was repeated using a more stringent sterilization protocol as well as incorporating the antibiotic PPM to all media. This reduced contamination to 17% in *M. punctata* and 33% in *M. fistulosa* (data not shown).

2.3.2 Evaluation of Lamina, Petiole and Nodal Tissues as Explants

When examining the response of the various explant tissues to *in vitro* propagation there was a marked difference for both *M. punctata* and *M. fistulosa.*

In *M. punctata* the lamina and petiole explants both showed a lower ability to regenerate (P=0.8926; Table A.1), while the nodal sections had a significantly greater number of plantlets than either lamina or petioles (P=<.0001; Table A.1). Unlike the nodal sections, both the lamina and petioles appeared to follow a trend of increasing the average number of shoots produced as

the concentration of BAP increased, producing the greatest number of shoots when exposed to 25µM BAP (0.7 and 0.9 shoots per explant, respectively; Figure 2.1). The linear regression further supported the increasing trend that was observed with a positive R^2 value of 0.1831 (data not shown). Although the percentage of explained variation was low, the model was significant (P=0.0001; data not shown). These values are based on the number of remaining, uncontaminated explants at the end of 12 weeks and represent the average number of shoots produced per explant tissue. There was a large variability in the regeneration capacity of individual explants observed. Often one explant would be healthy and highly regenerative, while other explants on the same petri plate turned brown and died with no shoot regeneration (Figure 2.2 A, C, E).

The explants of *M. fistulosa* had a similar response to those of *M. punctata*; however both the lamina and petiole explants of *M. fistulosa* were not as regenerative and the average number of shoots produced per explant was very low (data not shown). The majority of lamina and petiole explants turned brown or yellow and never produced new plantlets (Figure 2.2 B, D, E).

2.3.3 Effect of Light on Nodal Regeneration

The nodal sections used for the initial optimization experiments were exposed to a 24 hour light regime while the lamina and petiole explants were placed in a 16 hour light, 8 hour dark cycle. In order to determine if the light regime affected the regenerative ability of the nodal sections, new nodal sections were placed onto medium containing 25µM BAP and placed into a 16h photoperiod. *M. punctata* was not significantly affected by the hours of light it was exposed to, however it did produce more shoots when given an 8 hour night cycle (P=0.3400; Table A.2; Table 2.1). *M. fistulosa* however, did produce significantly more shoots in the 16/8 light cycle

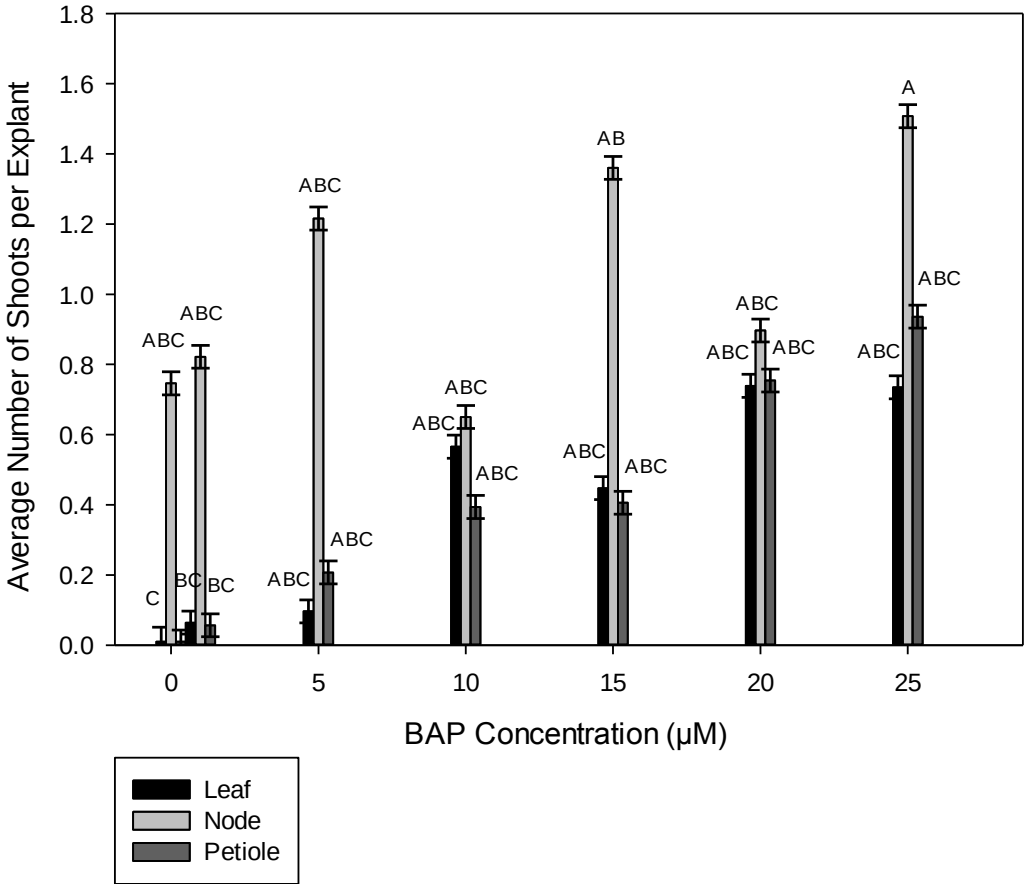

Figure 2.1. Average number of shoots produced by three explant tissues of *M. punctata* exposed to varying concentrations of 6-Benzylaminopurine. ***a-c*** Treatment means following the same letter are not significantly different (α=0.05) based on a Tukey's Means multiple range test.

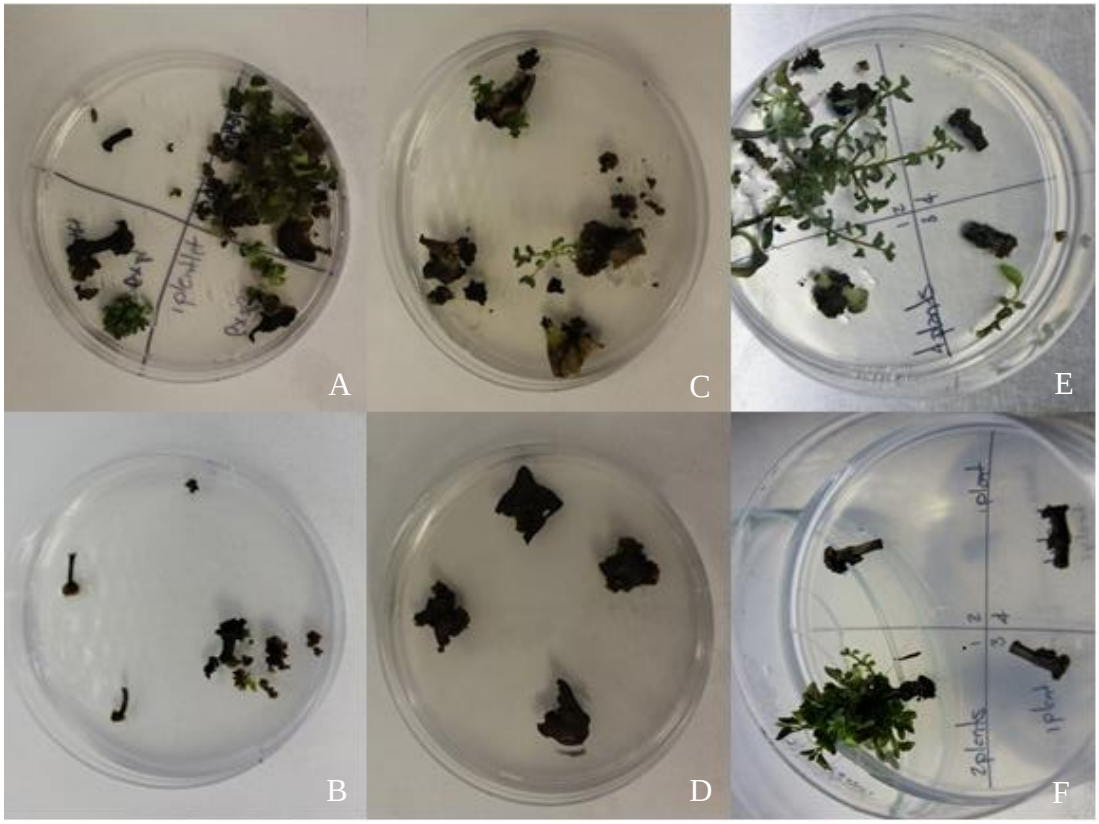

Figure 2.2. Extreme variation in the *in vitro* regeneration of *M. punctata* (A, C, E) and *M. fistulosa in vitro* (B, D, F) after 3 months on MS media supplemented with 10µM 6-Benzylaminopurine. A and B are petiole explants; C and D are leaf explants; E and F are nodal explants.

Table 2.1. Effect of photoperiod on the average number of shoots produced by *M. punctata* and *M. fistulosa* after 3 months on MS media supplemented 25µM 6-Benzylaminopurine.

Photoperiod (hour)	Average number of shoots	
	M. punctata	*M. fistulosa*
16	2.9*a*[z]	3.9*a*
24	1.5*a*	1.3*b*

[z] Treatment means following the same letter are not significantly different (α=0.05) based on a Tukey's Means multiple range test.

than in continuous light (P=0.013; Table A.3; Table 2.1).

2.3.4 Optimizing BAP Concentration

After evaluation of the three tissues it was determined that nodal sections were the superior explant source. Nodal explants became the focus for testing seven levels of BAP on proliferation of *M. punctata* and *M. fistulosa*.

It was observed both between replications and repetitions, that there was a large variability for individual nodal sections in their abilities to produce new plantlets (Table B.1-B.2). Four parameters were examined in order to compensate for the apparent differences between nodal sections.

Concentration of BAP had no significant effect on any of the regeneration parameters of *M. punctata* (Table A.4-A.7). Likewise, none of the linear models tested were found to be significant (data not shown). All four of the R^2 values were positive, however they were also very small (0.0-0.7; data not shown).

There were no significant differences observed among treatments for the average number of shoots (Table A.4). An average of 1.5 shoots per explant [1] was obtained on medium containing 25µM BAP, while the lowest average (0.3 shoots per total number of explant) was produced by nodes exposed to 10µM BAP (Figure 2.3; Table 2.2). This low rate of regeneration was accompanied by a high variability of the individual nodal sections to actually produce new plantlets (Figure 2.2 E).

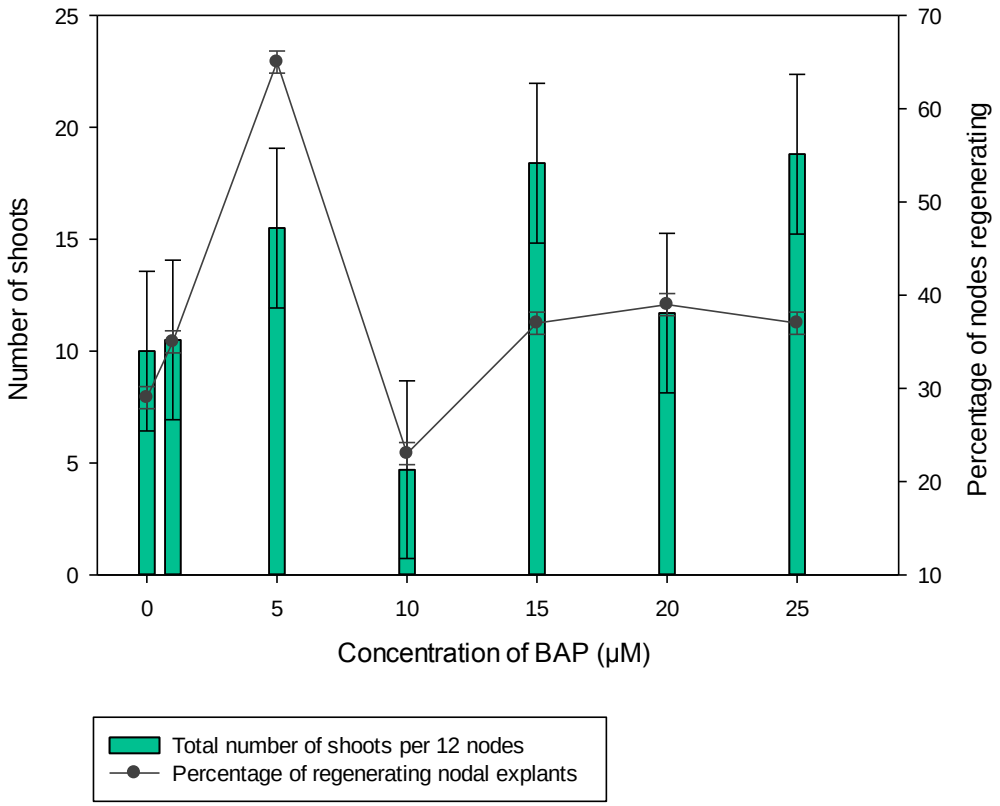

Figure 2.3. Total number of shoots produced by 12 explants of *M. punctata* in 12 weeks as compared to the percentage of explants regenerating over 12 weeks.

Table 2.2. Evaluation of *M. punctata's* ability to regenerate new plantlets when exposed to varying levels of 6-Benzylaminopurine for 12 weeks.

BAP (µM)	Average number of shoots per node	Percentage of nodes producing shoots	Average number of shoots per regenerative node	Total number of shoots produced by 12 nodes
0	0.7*ab*[z]	29*a*	2.8*a*	10.0*a*
1	0.8*ab*	35*a*	2.3*a*	10.5*a*
5	1.2*a*	65*a*	2.4*a*	15.5*a*
10	0.3*b*	23*a*	1.8*a*	4.7*a*
15	1.4*a*	37*a*	3.8*a*	18.4*a*
20	0.9*ab*	39*a*	2.3*a*	11.7*a*
25	1.5*a*	37*a*	4.4*a*	18.8*a*

[z] Treatment means following the same letter in the same column are not significantly different (α=0.05) based on a Tukey's multiple range test.

There was variability amongst the seven BAP concentrations in the percentage of nodes which were able to proliferate [2] (Table 2.2). On 5µM BAP, 65% of nodal explants developed shoots which was significantly higher than those sections on the control medium (P=0.0240, Table A.5).

When only the explants still alive at week 12 were considered, higher BAP concentrations continued to produce the greatest number of shoots. However, once again, no significant differences were observed (Table A.6). The average number of shoots originating from nodal sections on 25µM BAP increased from 1.5 shoots per total nodal section to 4.4 shoots per living nodal section and those sections on 10µM BAP rose from 0.3 to 1.8 shoots per explant (Table 2.2). These two treatments once again represent the highest and lowest average number of shoots.

In order to account for the varying number of explants which survived 12 weeks without becoming contaminated, the total number of shoots produced by the 12 nodal sections initially plated was calculated [4]. These numbers confirmed that the nodal sections of *M. punctata* produced the highest number (18.8) of new plantlets when exposed to 25µM BAP, while those in 10µM BAP produced the least amount (4.7) (Figure 2.3; Table 2.2), although differences were not statistically significant (Table A.7).

M. fistulosa had a similar response to that of *M. punctata*; however, higher rates of regeneration were obtained. There were no significant effects due to concentration (Table A.8-A.11) and there were no significant differences found between levels of BAP and the control (Table A.8-A.11) for any of the measured parameters in *M. fistulosa*. For the linear regressions tested, all R^2 values were once again small but positive (0.03-0.16) and only the linear model

applied to the average number of shoots per node was found to be significant (P=0.0359; data not shown).

The highest average number of shoots (3.3 and 3.4) per total nodal section was obtained from explants on 20 and 25µM BAP, respectively (Table 2.3). Nodes on 0µM and 5µM had the lowest rate of regeneration with an average of 1.5 shoots per total node (Table 2.3).

When the percentage of living explants was examined, explants on 20µM had 92% and 10µM or 25µM had 80% explants produce shoots, respectively (Figure 2.4; Table 2.3). The lowest percentage (28%) of regeneration was obtained from explants placed on medium with 15µM of BAP (Figure 2.4; Table 2.3). When only these explants were considered a similar trend once again emerged, with the highest average number of shoots per regenerative nodal section (4.2) being attained by tissues exposed to 25µM BAP, and the lowest average (2.3) by tissue placed in the control medium with no PGR added (Table 2.3).

The total number of shoots produced by 12 explants was calculated [4] and nodes on 25µM BAP once again produced the greatest number of shoots. Twelve nodes on 25µM BAP would have produced a total of 26.2 new plantlets, while the same number of nodal sections would only produce 18.4 new plantlets when placed on medium supplemented with 5µM BAP (Figure 2.4; Table 2.3).

In summary, the effect of BAP concentrations on the regeneration of nodal explants lacked significance and there were few significant differences observed between treatments and the controls. However, both species followed a similar trend and had the greatest regenerative capacity at the higher concentrations of BAP used.

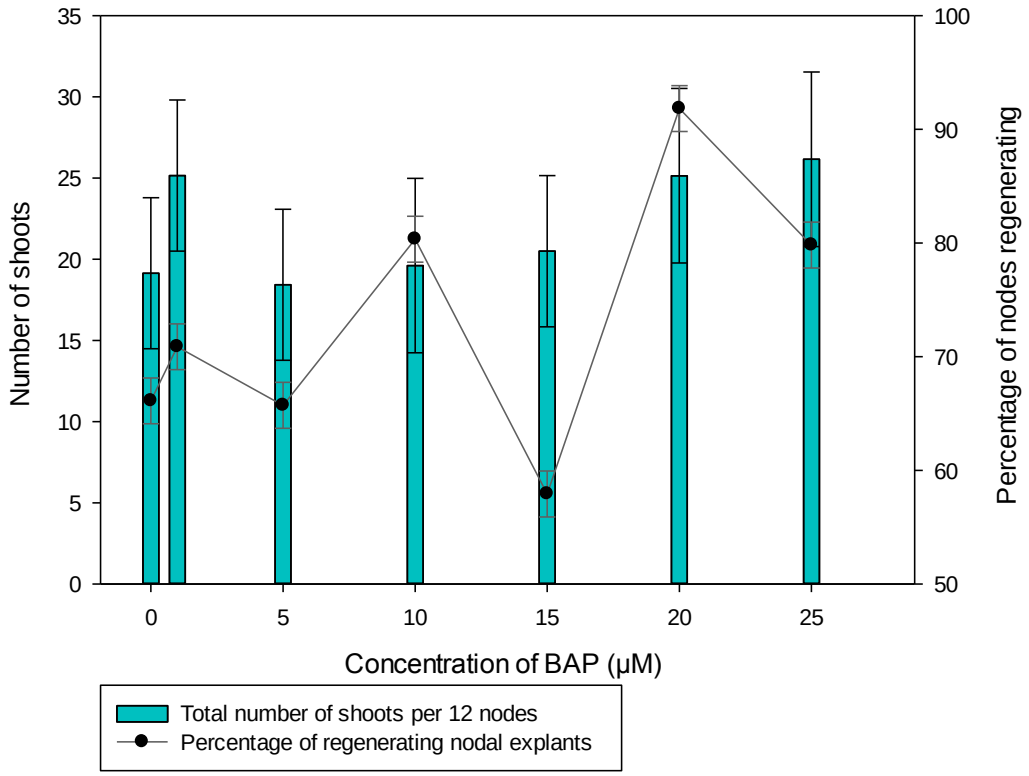

Figure 2.4. Total number of shoots produced by 12 explants of *M. fistulosa* in 12 weeks as compared to the percentage of explants regenerating over 12 weeks.

Table 2.3. Evaluation of *M. fistulosa's* ability to regenerate new plantlets when exposed to varying levels of 6-Benzylaminopurine for 12 weeks.

BAP (µM)	Average number of shoots per node	Percentage of nodes producing shoots	Average number of shoots per regenerative node	Total number of shoots produced by 12 nodes
0	1.5*a*[z]	66*a*	2.3*a*	19.1*a*
1	2.0*a*	71*a*	3.1*a*	25.2*a*
5	1.5*a*	66*a*	2.4*a*	18.4*a*
10	2.0*a*	80*a*	2.6*a*	19.6*a*
15	1.7*a*	58*a*	3.1*a*	20.5*a*
20	3.3*a*	92*a*	3.8*a*	25.1*a*
25	3.4*a*	80*a*	4.2*a*	26.2*a*

[z] Treatment means following the same letter in the same column are not significantly different (α=0.05) based on a Tukey's multiple range test.

2.3.5 Positional and Genotypic Effects

In all of the experiments conducted there was a high level of variability associated with the performance of individual nodal sections. Some died while others on the same petri plated produced several shoots. For both species, the individual genotype selected from the population had a significant effect on the number of plantlets produced, while the position of the nodal explant did not (Table A.12-A.15).

2.3.6 Rooting

There were few significant differences observed between individual treatments as determined by a Tukey's pairwise comparison in *M. punctata* (Table A.16-A.19; Table 2.4). The presence of activated charcoal (AC) in the media was associated with an increase in both the total number of roots produced (P= 0.0334; Table A.16) and the length of the longest root in *M. punctata* plantlets (P= 0.0327; Table A.17). However, the ½ MS had no effect on the total number of roots and root length (P= 0.3814; 0.8992; Table A.16-A.17). This is in contrast to the significant increase in height and node number when *M. punctata* plantlets were on full strength MS medium (P=0.353; 0.0065; Table A.18-A.19), while the presence or absence of AC had no effect on the vegetative growth of the plantlets. The only significant differences between the treatments for the traits measured were the ability of plantlets on MS + 2.5µM IBA to produce more roots than on the control MS, and plantlets on MS + 5.0µM IBA produced a greater change in height than those placed on to either ½MS + 5.0µM IAA or the control medium (Table 2.4). All roots produced by all treatments were classified as very thin and fibrous (Fig 2.5).

Table 2.4. Effects of various rooting media treatments on root and shoot characteristics of *M. punctata* plantlets after a six week culture period.

Treatment (µM)	Average number of roots	Average length of longest root (cm)	Average change in plantlet height (cm)	Average change in plantlet nodes
MS	0.7*b*[z]	0.9*a*	2.7*b*	3.2*a*
MS + 2.5 IBA	4.6*a*	1.4*a*	4.3*ab*	2.8*a*
MS + 5.0 IBA	3.3*ab*	1.1*a*	6.9*a*	4.3*a*
MS + 5.0 IAA	1.3*ab*	1.2*a*	3.8*ab*	3.9*a*
MS + ac	2.5*ab*	2.7*a*	5.7*ab*	3.9*a*
MS + ac + 2.5 IBA	2.9*ab*	1.6*a*	4.0*ab*	3.8*a*
MS + ac + 5.0 IBA	3.8*ab*	2.6*a*	5.4*ab*	3.9*a*
½MS	1.1*ab*	2.2*a*	4.6*ab*	3.6*a*
½MS + 5.0 IAA	1.4*ab*	1.3*a*	2.1*b*	2.0*a*
½MS + ac	3.6*ab*	1.9*a*	2.8*ab*	2.3*a*
½MS + ac + 2.5 IBA	3.3*ab*	2.5*a*	3.6*ab*	2.5*a*
½MS + ac + 5.0 IBA	3.9*ab*	2.0*a*	3.0*ab*	2.3*a*
½MS + ac + 7.5 IBA	3.5*ab*	2.1*a*	4.5*ab*	2.8*a*
½MS + ac+ 10.0 IBA	4.8*a*	2.1*a*	5.3*ab*	4.0*a*

[z] Treatment means following the same letter in the same column are not significantly different (α=0.05) based on a Tukey's multiple range test.

Figure 2.5. Example of thin, fibrous roots produced by *M. punctata* on a basal MS medium for six weeks.

M. fistulosa was more responsive to the rooting treatments that *M. punctata.* In *M. fistulosa* the total number of roots were significantly affected by the presence or absence of AC (P= <.0001; Table A.20) and there was a further decline in root production when AC was coupled with a ½ MS basal medium (Table 2.5). While treatment did have a significant effect of the length of the longest root (P= <.0001; Table A.21), both AC and the MS salt strength did not have significant effects (P= 0.9783; 0.4726; Table A.21). Roots produced by *M. fistulosa* were less fibrous than those from *M. punctata*; however they were still relatively thin and weak.

Vegetative growth was favoured in basal MS medium with no AC (Table 2.5). ½ MS and AC had an inhibitory effect on height and node number (P= <.0001; <.0001; Table A.22-A.23).

Based on all the factors evaluated, it is believed that a ½ strength basal MS medium with no additional supplementation would be optimal for the *in vitro* rooting of *M. fistulosa.* Plantlets on this medium had the greatest number of roots (10.7) and the longest root (4.1cm) while experiencing the fifth greatest increase in height (11.0cm) and node number (3.4; Table 2.5). This is in contrast to *M. punctata* where ½ MS + AC + 10µM IBA or MS + AC + 5µM IBA were optimal for *in vitro* rooting. The first medium produced the greatest number roots (4.8) and the second longest root (2.6cm), while still producing a height increase of 5.3cm and 4.0 nodes (Table 2.4). Plantlets on the second medium however, produced an average of 3.8 roots with the greatest length (2.6cm) with an increase of 5.9cm in height and 3.9 nodes (Table 2.4).

2.3.7 Acclimatization

Due to the poor *in vitro* rooting, plantlets were transferred to the greenhouse as unrooted plantlets. Both species had sufficient rates of adaptation to the greenhouse environment. *M.*

Table 2.5. Effects of various rooting media treatments on *M. fistulosa* plantlets after six week culture period.

Treatment (μM)	Average number of roots	Average length of longest root (cm)	Average change in plantlet height (cm)	Average change in plantlet nodes
MS	5.7 *c*[z]	3.4 *ab*	11.7 *ab*	3.9 *a*
MS + 2.5 IBA	5.6 *cd*	1.8 *bc*	11.4 *ab*	3.9 *a*
MS + 5.0 IBA	3.9 c*de*	1.5 *c*	12.6 *a*	3.7 *ab*
MS + 5.0 IAA	7.9 *abc*	3.7 *abc*	11.5 *ab*	3.6 *abc*
MS + ac	6.7 *bc*	3.4 *ab*	10.2 *ab*	3.2 *abc*
MS + ac + 2.5 IBA	6.0 *c*	3.9 *a*	10.2 *ab*	3.3 *ab*
MS + ac + 5.0 IBA	6.3 *c*	3.3 *ab*	9.2 *bd*	2.8 *abc*
½MS	10.7 *a*	4.1 *a*	11.0 *ab*	3.4 *abc*
½MS + 5.0 IAA	9.8 *ab*	3.2 *abc*	9.3 *abc*	2.8 *abcd*
½MS + ac	2.2 *de*	2.7 *abc*	4.9 *ce*	1.6 *cd*
½MS + ac + 2.5 IBA	2.1 *e*	2.5 *abc*	3.3 *e*	1.0 *d*
½MS + ac + 5.0 IBA	3.8 *cde*	3.2 *abc*	5.1 *ce*	2.2 *bcd*
½MS + ac + 7.5 IBA	1.6 *e*	1.8 *bc*	3.3 *e*	1.6 *cd*
½MS + ac+ 10.0 IBA	3.8 *cde*	2.9 *abc*	5.0 *cde*	1.5 *cd*

[z] Treatment means following the same letter in the same column are not significantly different (α=0.05) based on a Tukey's multiple range test.

Figure 2.6. Example of thin roots produced by *M. fistulosa* on a basal MS medium for six weeks.

punctata had an 81% survival rate, while *M. fistulosa* demonstrated an 87% survival rate following acclimatization. There was no effect of initial BAP treatment on the success of acclimatization into the greenhouse (data not shown).

2.4 Discussion:

2.4.1 Contamination

The increased contamination rates of the nodal section could be due to increased trichome presence on the stem as compared to leaf or petiole tissues (Smith, 2012; Pinto *et al.*, 2012). In the current investigation an increased concentration of bleach, longer exposure to the bleach and ethanol, the introduction of a pre wash in running water, as well as the addition of the antimicrobiotic PPM into the basal medium, the rates of contamination were reduced to an acceptable range for nodal sections. PPM has been shown in several other species to be a highly effected antibiotic and aids in reducing contamination (Niedz and Bausher, 2002; Ruta and Morone-Fortunato, 2010).

2.4.2 Comparison of Explants

The present study examined the efficiency of regeneration from three different explant sources: lamina, petiole and nodal with the objective of obtaining new plantlets through direct regeneration or proliferation. The selection of explant tissue has long been regarded as a crucial first step for the successful initiation of new *in vitro* cultures (Smith, 2012).

The plant tissue used as an explant donor can affect the outcome and success of *in vitro* propagation in various ways. The culturabilty of any explant source has been shown to exhibit large variability from species to species. Several studies have examined the effect of explant

source during the development of *in vitro* propagation systems. Kulkarni *et al.* (2000) examined direct regeneration from the nodes, internodes, hypocotyls and embryos of *Withania somnifera* (L.) Dun. (Ashwagandha). They found that embryos were unable to regenerate at any concentration of BA, both nodes (14.0 shoots) and hypocotyls (12.9 shoots) reached an optimal regeneration when exposed to 2.2µM BA and internodes placed on 22.2µM BA were able to regenerate over 40 shoots (Kulkarni *et al.,* 2000). This study demonstrated the variability between explant sources as well as highlighting the difference in PGR optimum that can be associated with individual explant sources. These findings are also supported by a study conducted in *Bacopa monniera* (L.) Wettst (Brahmi) with nodes, internodes and leaf discs as potential explant donors (Tiwari *et al.,* 2006). Various explants had different rates of regeneration that were reached with various levels of BA with both nodal sections (20.6 shoot buds/explant) and internodes (30.1 shoot buds/explant) producing the greatest number of shoots on a medium containing 6.7µM BA while leaf discs had the greatest rate of regeneration (79.7 shoot buds/explant) when exposed to 8.9µM BA (Tiwari *et al.,* 2006).

The selection of explant can also affect the method of regeneration. Meristem, shoot and nodal cultures are all more likely to produce new plantlets through direct organogenesis (Smith, 2012). This is due to the fact that these tissues already have auxiliary meristems present and simply need the correct hormonal balance to overcome any apical dominance which may inhibit growth. This is contrast to tissues such as leaves, petioles and stem sections that are more likely to regenerate indirectly through the production of callus (Smith, 2012), although direct regeneration has been reported (Detrez *et al.,* 1988).

Within Lamiaceae, species have been successfully propagated from leaves, nodes, cotyledons and shoot tips (Dode *et al.,* 2003; Pattnaik and Chand, 1995; Kintizios *et al.,* 2004;

Maity *et al.*, 2011; Dube *et al.*, 2010). Although regeneration has been successfully demonstrated in each of these explants, nodal sections and auxiliary buds have been more widely used and accepted as an explant donor within the family, demonstrating successful regeneration in: *Ocimum basilicum* (Kintizios *et al.*, 2004), *Cunila galioides* (Fracaro and Echeverrigaray, 2000), *Hyssopus officinalis* (Toma *et al.*, 2004), *Lavandula pedunculata* (Zuzarte *et al.*, 2010) and *Coleus forskohii* (Dube *et al.*, 2010).

2.4.3 Optimizing BAP Concentrations

BAP was selected as the PGR for several reasons. First, it is an effective regulator of regeneration within Lamiaceae and is commonly used without the addition of auxins (Maity *et al.*, 2011; Sahoo *et al.*, 1996). When examined concurrently with other cytokinins, BAP often outperformed others (Dube *et al.*, 2010; Fracaro and Echeverrigaray, 2000). Second, it has been shown to be highly efficient at releasing auxiliary bud dormancy previously suppressed through apical dominance (Fracaro and Echeverrigaray, 2000), leading to the formation of new shoots. While the use of this cytokinin is common within Lamiaceae, an optimal range of 0.22-4.4µM BAP has been previously cited (Fracaro and Echeverrigaray, 2000). However, in the current study, regeneration rates tended to increase at higher concentrations of BAP and 25µM was selected as the optimum concentration for regeneration. This was determined by assessing the data using four related, but distinct parameters. In each of these parameters there was a small, non-significant, but consistent upward trend towards 25µM BAP with no visible plateau or peak being obtained, indicating that a wide range of BAP concentrations could be used with these species.

2.4.4 Preliminary Effect of Genotype and Nodal Position on *in vitro* Regeneration

This lack of significance is thought to have been caused by some extraneous factors which were not expected. The donor explant material was from *in vivo* greenhouse grown plants which originated from seed collected from an outcrossing population. This resulted in a population of donor plants with a diverse genetic background. Plants were randomly sampled for induction into cultures so there were likely genotypic differences between the individual explants. Another possible source of variation could be attributed to the position and age of the explant that was taken.

Genotype has been shown to affect the ability of plants to adapt to and thrive in *in vitro* environments. A study conducted in sugar beet (*Beta vulgaris* L.) examined five different breeding lines for their ability to regenerate new plantlets from petiole cultures placed on the same medium (Detrez *et al.,* 1988). The genotypes varied significantly in their ability to form buds, with 70% of petioles from line SW108 able to form buds, while only 20% of petioles from the M2 line produced new buds (Detrez *et al.,* 1988). Another study using four ecotypes from *Arabidopsis thaliana* once again found considerable variation for leaf explant regeneration rates for the different ecotypes (Candela *et al.,* 2000). Within those ecotypes, Landsberg *erecta* and Wassilewskija demonstrated the highest regenerative callus percentages of 56% and 75%, respectively, while Cape Verde Island and Columbia had much lower rates of 27% and 3% respectively (Candela *et al.,* 2000).

A second cause of the variability between individual explants can be attributed to the age and position of the nodal sections at time of collection. As with the selection of the individual donor plants, 5-10cm sections of healthy stem were randomly selected in order to initiate

cultures. The tissue age differences within these sections may have added to the variably in regeneration efficiency, as there is often considered to be an optimal developmental age for the donor tissue to produce the most regenerative explants. This effect of explant age has been documented in other *in vitro* systems. Dhar and Joshi (2005) found that explant donor 5 days old were too young and thin to survival culture and would often wither and die before producing callus, while 20 day old tissue donors were considered to be too advanced in development and ultimately were harder to regenerate. It has been suggested that a contributing factor to the effect of explant is age. Young tissue is thought to be more responsive *in vitro* (Smith, 2012), and have more metabolically active cells which may result in these cells possessing more nutritional components and endogenous hormones, both of which aid in increased regeneration (Dhar and Joshi, 2005).

With these *Monarda* species, both genetics and nodal age, may have contributed to the extraneous variation in the regeneration rates between treatments in an unexpected and unplanned manner. The effect of BAP concentration was often masked by large amounts of explant to explant variation which ultimately made it difficult to measure the effect of BAP concentration. It is believed that the seemingly low rates of regeneration in the current investigation could be improved by optimizing the age of the *in vitro* plantlets and tissue for explant collection, as well as other conditions such as light and fertilizer.

The results from the current study lead to the same conclusions drawn by Detrez *et al.* (1988) and Candela *et al.* (2000) that genotype does significantly affect the regenerative ability of explant *in vitro* and can create large variability within cultures.

2.4.5 Rooting

The *in vitro* rooting of both plant species was considered to be somewhat difficult and less than optimal. Large arrays of rooting media were tested in order to help overcome these difficulties; however none proved to be efficient in rooting *M. punctata in vitro*. Other members of the family have been reported to have a greater success in *in vitro* rooting. A study conducted in Mint (*Mentha arvensis* Linn), a member of the Lamiaceae family, was able to attain 9.2 roots per explant using a MS medium supplemented with 4.9µM IBA after 25 days (Maity *et al.,* 2011).

The use of AC in rooting mediums has previously been documented to aid in root production. Gantait *et al.* (2009) examined the effect of three concentrations of AC on the root induction and development of *Dendrobium chrysotoxum* grown *in vitro.* The addition of AC at a concentration of 2g/L produced significantly higher root numbers (4.3) than medium supplemented with only 1g/L (2.7 roots) or no AC (1.7 roots) (Gantait *et al.,* 2009). AC may aid in the production of roots by binding and detoxifying media from inhibitory phenolics released by the plantlet *in vitro* (Gantait *et al., 2009).* AC may also help to remove some PGRs applied during shoot regeneration which can inhibit the formation of roots (Rout *et al.,* 2006)**.**

Similarly, the reduction in vigor that was associated with *M. punctata* plantlets placed onto media supplemented with ½ the strength of the basal MS salt base has been previously documented. Fracaro and Echeverrigaray (2000) demonstrated this in *Cunila galioides* by comparing a full strength, ½ strength and ¼ strength MS salt base. Plantlets on a ½ and ¼ strength MS basal medium were significantly shorter (2.2 and 2.3 cm, respectively) as compared to those on a full strength medium (3.4 cm)**.** This reduced vigor could be caused by the reduction

of nitrogen. It is possible that the plantlets exhausted the reduced nutrients in the ½ MS and nutrient availability was the cause of the limited vegetative growth observed. However, the reduction in height did not result in a subsequent reduction of root formation (Fracaro and Echeverrigaray, 2000).

M. fistulosa attained better rooting compared to *M. punctata*; however these numbers were still lower than previously published reports. Plantlets from *M. fistulosa* which were exposed to media containing both AC and a reduced salt base experienced a negative additive effect on both vigor and root production. This combination may have caused a reduction of available nutrients. As previously noted, a reduction in MS salt concentration can result in a reduction of nitrogen available to the growing plantlet. This reduction could be exacerbated by the presence of AC, which will bind PGRs, phenolics, as well as nutrients present within the media. Overall, this combination may further reduce available nutrients for the growing plantlet and ultimately reduce its total vigor.

While the combination of these two treatments resulted in a decline in growth and vigor, the best rooting medium tested for *M. fistulosa* was determined to be a basal ½ MS medium with no additional supplementation. This rooting medium has previously been reported to have mixed success for *in vitro* rooting. Sahoo *et al.* (1996) reported that shoots of *Ocimum basilicum* failed to produced roots when placed on ½ MS with no additional hormones. However, another study by Dode *et al.* (2003) also using *Ocimum basilicum* placed regenerated shoots onto ½ MS media without growth regulators for 45 days and observed root formation with successful transfer to the greenhouse. The reduced available nutrients may support root formation by promoting the production of root number and length. In *ex vitro* conditions, this would be beneficial for a plant in order to find more available nutrients within the rhizosphere.

2.4.6 Acclimatization

The results presented here showed a high percentage of plantlets successfully acclimatized to greenhouse conditions. Previous studies have attained successful acclimatization rates of: 88% in *Ocimum basilicum* (Sahoo *et al.,* 1996), 89% in *Cunila galioides* (Fracaro and Echeverrigaray, 2000) and 90% in *Mentha arvensis* Linn. (Maity *et al.,* 2011). The use of Stimroot #1 and directly placing plantlets into a soil substrate is considered a desirable trait for *in vitro* propagated ornamental plant systems. The ability to forgo an *in vitro* rooting phase can eliminate six to eight weeks of culture time and labour costs from the regeneration scheme. Both species of *Monarda* were able to successfully acclimatize to greenhouse conditions with an application of Stim Root #1 and very few to no *in vitro* roots.

2.5 Summary:

In summary, both *M. punctata* and *M. fistulosa* can successfully be propagated *in vitro* through nodal sections using the cytokinin BAP as the only PGR, or in the absence of any additional PGR. While 25µM of BAP was chosen as optimal for continued studies, it was difficult to discern an optimal medium due to the high amounts of variability caused by the heterogeneous donor population. Several media were tested in order to induce *in vitro* rooting. While some roots were produced, the root system lacked vigor and were not considered optimal and further development is needed in order to successfully induce better root production *in vitro*. However, these plantlets did successfully elongate when treated with Stim Root #1 and transplanted directly into a soil substrate. The transplants were able to acclimate to greenhouse conditions with a high rate of survival. Future work to further optimize the regeneration system might be aimed at studying the effects of tissue age and nodal position in the two *Monarda*

species in more depth. Rooting *in vitro* was weak and deserves attention although the current protocol did result in good survival during acclimation.

3.0 Chapter 3: Development and optimization of protocols to induce polyploidy for *M. punctata* and *M. fistulosa in vitro* and verification of autotetraploids through cytological studies

Abstract:

Tetraploids of both *M. punctata* and *M. fistulosa* were successfully created through the application of trifluralin or oryzalin *in vitro* across a range of concentrations. *M. punctata* showed a decrease in survival as duration of exposure increased. However, this trend was not observed in the percentage of polyploidization which was largely unaffected by exposure length. This was in contrast to *M. fistulosa* in which survival and number of polyploids were negatively affected by increasing duration. *M. punctata* produced more tetraploids and had a higher rate of survival when trifluralin was applied while *M. fistulosa* did not demonstrate a preference. Neither species were strongly affected by the concentration of the anti-mitotic agent applied.

3.1 Introduction:

Two species of the genus *Monarda*, members of the family Lamiaceae, are valued not only for their ornamental potential but also their medicinal properties (Johnson et al., 1998; Scora, 1967b; Yamada et al., 2010; Zhilyakova, *et al.*, 2009). *M. punctata* and *M. fistulosa* are members of the *Monarda* genus native to Ontario; however, while these two species share a genus, they do not belong to the same sub-genera (Anderson 2000 & 2003). *M. punctata* is a member of the sub-genera *Cheilyctis* while *M. fistulosa* belongs to the sub-genera *Monarda* (Scora, 1967a). One of the distinctions between these species is their chromosome numbers. *M. punctata* has a base chromosome number of 11 (2n=2x=22), while *M. fistulosa* has a base number of 18 (2n=2x=36) (Scora, 1967a). Both species have been the subject of published

investigations into medicinal uses for their essential oils as well as isolating individual compounds from the oil (Scora, 1967b; Yamada *et al.,* 2010; Johnson *et al.,* 1998). *M. punctata's* oil is thought to contain compounds which help with inflammation (Yamada *et al.,* 2010) while *M. fistulosa's* oils are being investigated for potential cancer fighting properties (Johnson *et al.,* 1998).

M. punctata and *M. fistulosa* have recently been the focus of studies to develop *in vitro* propagation systems. Reports indicate that these species can be successfully propagated *in vitro* and acclimatized to greenhouse conditions using a very basic medium and no additional steps for rooting (Chapter 2). By modifying this system through the addition of anti-mitotic agents in the medium it may be possible to create synthetic autotetraploids. There are several reasons that the creation of autotetraploid *Monarda* cytotypes is of interest, including potential increases to ornamental characters, drought tolerance and essential oil concentration. The decision to create these plantlets using *in vitro* techniques was based on reports of higher rates of success as well as a more stable ploidy level when applied *in vitro* vs *in vivo* (Zhang *et al.,* 2008).

Induced autopolyploidy has produced interesting results. Most studies agree that generally, an increase in ploidy level results in an increased plant size, larger leaves with a darker colour, and larger flowers with more intense colouring (Allum *et al*., 2007; Emsweller and Brierly, 1940; Emsweller and Ruttle, 1941; Gu *et al*., 2005; Kermani *et al.,* 2002; Kobayashi *et al*., 2008; Van Laere *et al.,* 2011; Leven, 1940; Rose *et al*., 2000). There have also been a few investigations which have examined the physiological effects of chromosome duplication. These studies have reported an increased drought tolerance and heat tolerance and an increased resistance to pests (Arseniuk, 1989; Emsweller and Ruttle, 1941; Li *et al.,* 1996; Warner and Edwards, 1989). A relationship between stomatal density, net photosynthetic rates under water

stress and ploidy level was also reported in *Spathiphyllum wallisii* (Van Laere *et al.,* 2011). An increase in chromosome number has also led to increased volumes and altered chemical profiles of essential oils. A study in *Braza media* reported different chemical constituents of diploid essential oils and tetraploid oils (Murray and Williams, 1976). Another study conducted using naturally derived cytotypes of *Cymbopogon flexuosus* (lemon grass) reported a positive correlation between genome copy number and essential oil concentration (Janaki-Ammal and Gupta, 1996).

Anti-mitotic agents are derived naturally (colchicine) and synthetically (trifluralin, oryzalin) and act on cells undergoing division through mitosis (Ascough *et al.,* 2008). These chemicals have been found to disrupt the formation of the spindle fibers at metaphase by binding to the tubulin dimers which form the fibers (Dhooghe *et al.,* 2009). This results in a single cell which has already undergone DNA replication in preparation for division; however without the formation of spindle fibers to separate the sister pairs of chromosomes and form the metaphase plate, division fails to actually occur, known as endoreduplication (Ascough *et al.,* 2008; Dhooghe *et al*., 2009). The cell which now has a doubled compliment of chromosomes will arrest into G phase, and then continue with normal division and proliferate into a complete new *in vitro* plantlet, as well as possibly giving rise to a new cell linage(s) and subsequent plantlet(s) (Ascough *et al.,* 2008; Dhooghe *et al*., 2009). These resulting plantlets are known as synthetic autotetraploids.

Autopolyploidy means that the individual has multiple copies of the same genome derived by duplication of its own chromosomes (i.e. genome AA is copied, resulting in the autotetraploid AAAA) (Dhawan and Lavania, 1995). However, another form of polyploidization known as allopolyploidy results in an individual with multiple and different genomes introduced

through breeding or biotechnological efforts (Dhawan and Lavania, 1995). The result is an individual with a genotype of AABB where A and B are different genomes. This is actually a fairly common genome composition, with examples such as bread wheat (*Triticum aestivum*) being an allohexaploid (AABBDD), and strawberries (*Fragaria virginiana*) are considered auto/allooctaploids (AAA'A'BBB'B').

The three most commonly used anti-mitotic agents to induce autopolyploidy are colchicine, oryzalin and trifluralin. Colchicine is a naturally derived metabolite, originally extracted from *Colchicum autumnale* (Ascough *et al.,* 2008). This compound is very toxic for mammals, although small doses are used in the treatment of diseases such as gout (FDA, 2009). Oryzalin and trifluralin are synthetically developed compounds which are both used as the active ingredients in herbicides (Ascough *et al.,* 2008). While synthetically derived, these two chemicals have been reported to have better efficiency at binding to plant tubulins than colchicine with lower risk to human health (Dooghe *et al.,* 2009). They have also been reported to produce more stable polyploids with fewer mixoploid plants and fewer instances of ploidy reversion (Zhang *et al.,* 2008).

The objective of this investigation is to induce autotetraploidy in *M. punctata* and *M. fistulosa* using nodal sections subjected to different concentrations of oryzalin and trifluralin for varying durations *in vitro*. These autotetraploids will be identified using cytological methods and anatomical differences to differentiate them from their diploid cytotypes.

3.2 Materials and Methods:

3.2.1 Oryzalin and Trifluralin Stock Solutions

Stock solutions of both chemicals were made fresh the day of media preparation with a final concentration of 10mM. Powdered oryzalin or trifluralin (Sigma Alrich, Oakville, Ontario) was weighed with an analytical scale and dissolved in 15mL of 100% Dimethyl sulfoxide (DMSO) (Sigma Aldrich, Oakville, Ontario). The stock solutions were placed in 50mL polypropylene centrifuge tubes, wrapped in aluminum foil and kept at room temperature until used.

3.2.2 Preparation of Induction Media

The MS basal medium consisted of: MS minerals and vitamins, sucrose, plant preservative mixture (PPM) (Plant Cell Technology, Washington, USA) and 25µM 6-benzylaminopurine (BAP) (Sigma Alrich, Oakville, Ontario) as previously described (Chapter 2). Medium was brought to a final pH of 5.8, divided into eight 1000 mL Pyrex bottles containing 395.2 mL of medium and 7% lab grade agar (Fisher Scientific, Canada). All bottles were autoclaved at 104kPa and 121°C for 20 minutes. Upon completion of the autoclave cycles, bottles were removed, placed into a 60°C water bath and allowed to cool. Once bottles had cooled to the touch, the appropriate amounts of oryzalin or trifluralin stock solutions were added, along with subsequent DMSO volume in order to bring the final volume of DMSO added to all bottles to 4.8mL (Table B.1). Media was mechanically agitated to mix the chemical throughout and dispensed into 100mm x 25mm petri dishes to yield a total of 16 plates / 400 mL. Once media was cooled and solidified, it was packaged, sealed and stored in the dark at room temperature until the start of the experiment.

3.2.3 Induction of Tetraploidy

On the induction day, 48 *M. fistulosa* and 48 *M. punctata* plantlets were selected for plating. Prior to plating, plantlets were kept in 25 x 150mm culture tubes containing a MS medium and were maintained at 25°C ± 1°C in a 16 hours light and 8 hours dark regime.

For each petri plate, the apical portion of a stem containing 2-3 nodes was removed and placed into a culture tube containing fresh medium, leaving the remaining basal portion of the stem. The first four nodes immediately below the apical portion of the stem were then isolated and used for the experiment. The leaves and petioles were removed and the stem was sectioned into four 1cm long nodes. Each of the four nodes was then laid onto induction medium and gently pressed so approximately half the diameter of the node was embedded. The experiment was arranged in a randomized complete block design. Each block included six petri plates at each concentration of anti-mitotic agent, three made using oryzalin and three with trifluralin. Each of these three plates, after nodes were partially embedded, were randomly assigned one, three or six days of exposure to the anti-mitotic medium. A single block consisted of aforementioned procedures completed for each of the eight concentrations tested (0µM, 1µM, 5µM, 15µM, 30µM, 60µM, 90µM, 120µM) of both chemicals.

After either 1, 3 or 6 days of exposure to the induction medium, the nodes were removed and placed into fresh medium containing only MS, vitamins, PPM and 25µM BAP. All plates were sealed with parafilm and placed into a growth cabinet (25°C ± 1°C; 16 hour light and 8 hours dark). After the initial transfer from the induction media, nodal sections were transferred to fresh medium every four weeks. Any developing plantlets larger than 1.5 cm at the time of transfer were removed and placed into individual culture tubes containing autoclaved MS

medium, MS vitamins and 0.7% agar. Tubes were sealed with micropore tape and placed into growth cabinets and maintained under the same growth conditions as the petri plates (25°C ± 1°C; 16 hour light and 8 hours dark).

3.2.4 Acclimatization

At the end of the treatment period, plantlets were transferred to the greenhouse when plantlet height was greater than 15cm. Plantlets were removed from the culture tubes and all agar was gently washed from basal portion of the stem as well as from any developing roots. Roots and basal portion of the stem were dipped into Stim Root#1 powder and placed into 50 cell trays containing a 1:1 (v/v) ratio of sunshine mix #4 and turface. All trays were placed into a misting bed for two weeks, removed and placed onto a moisture mat and covered with plastic domes for an additional two weeks. Once rooted plantlets were established, they were transplanted to 8.9cm pots containing the same soil mixture and maintained at 24°C ± 2°C with 14 hours light and 10 hours dark.

3.2.5 Stomatal Measurements

Stomatal measurements were completed on all acclimatized, transplanted plantlets. The first fully expanded leaves of nine plants were harvested at one time. Leaves were cut approximately 0.5cm from the leaf tip into 1 cm X 1 cm squares. Leaf squares were placed abaxial side up onto a glass slide and water and cover slide were placed on top. Stomates were examined under brightfeild with either an Olympus Bx51 (Olympus Canada, Richmond Hill, ON) microscope or an Ancansco biological microscope (Ancansco, Toronto, ON) using a 60x optical lens. The length and width of four stomates were measured using a scale bar image taken

with each grouping of stomates and Image J (Abramoff *et al.*, 2004). The average of the four stomates per leaf was calculated for each plant.

3.2.6 Flow Cytometry

Flow cytometry was completed using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). New, healthy leaves were harvested from plants, wrapped in moist paper towels and sealed in Ziploc bags until used later that day. Leaf tissue of either *M. punctata* or *M. fistulosa* was placed in a Petri dish with approximately equal amounts of *Glycine max* Merr. 'Polanka' leaf tissue, 0.7mL of buffer was added and the tissue was finely chopped with a razor blade. The buffer was LBO1 (Doležel *et al.*, 1989) containing 50µg/mL of RNAse and 100µg/mL of propidium iodide (PI), however a subsample of tissue was run with only 50µg/mL PI. The leaf mash and buffer were then strained through a Celltrics 30µm filter (Sysmex America, Inc., Lincolnshire, IL, USA) and into a 5mL Falcon polystyrene tube (Fisher Scientific, Canada). Samples were placed in the dark at room temperature for a minimum of 20 minutes and a maximum of 50 minutes until analyzed with the flow cytometer.

All data were acquired and analyzed using CellQuest Pro software (Ver. 4.0.2, 2002, BD Biosciences, San Jose, CA, USA). FL2 area was used to measure relative fluorescence and to calculate relative DNA content. The integrated fluorescence values for signals were detected with the 485nm photodetector (Fig 3.1). *Monarda* nuclei peak means on the FL2 area histograms were compared to the peak means of the internal standard *Glycine max* Merr. 'Polanka' (2C = 2.5pg; Doležel *et al.*, 1994) and the 2C DNA content (pg/2C) of both *Monarda* species were found based on the relative fluorescence. The mean diploid peak and pg of DNA were confirmed using untreated plants of both species. These values allowed for the confirmation of ploidy level

for each plant tested. Plants which had two distinct peak formations at the tetraploid and diploid FL2 area were considered mixoploids.

Once a correlation between stomatal size and ploidy was determined using Spearman's rank, stomatal length data were used to group remaining plants with similar stomatal size into groups of 2-4 plants. To increase the efficiency of flow cytometry, small amounts of each plant's leaf tissue as well as soy leaf were combined, macerated in the buffer and used for analysis. If more than one peak formed, these plants would be re-run as separate plant samples.

3.2.7 Statistical Analysis

The effects of chemical, concentration and duration on regeneration *in vitro* were analyzed by totaling the number of shoots produced in each treatment across all four explants over four months. A Proc Mixed procedure was carried out using SAS 9.3 (SAS Institute, Inc., Cary, NC) and all values were compared using Tukey's pairwise comparison. A log transformation was performed in order to normalize the data as determined by the W value (Shapiro-Wilk) and all data were back transformed for reporting. In order to determine the relationship between ploidy level and stomatal length, ploidy was first given a rank: 1 for tetraploid; 0.5 for mixoploid and 0 for diploid, and then a Spearman's Rank correlation was performed using a Proc CORR procedure.

3.3 Results:

3.3.1 Effect of Chemical, Concentration and Duration on *in vitro* Regeneration

The main effects of chemical, concentration and duration were significant on the total number of plantlets produced by *M. punctata in vitro*. However there were no significant

interaction effects between these three variables so only the main effects are reported (Table A.24). Trifluralin produced significantly more plantlets (3.5 per explant) than Oryzalin (2.3 per explant) (Table 3.1). Duration of exposure for shorter periods (i.e. 1 day versus 6 days) resulted in significantly more plantlets (Table 3.1). A 1µM concentration produced the highest number of plantlets (5.3), and was significantly more regenerative than explants exposed to higher concentrations such as 60 or 90µM (1.4 and 1.5 plantlets, respectively) (Table 3.1).

For *M. fistulosa* there were no significant interactions between concentration, chemical or duration (Table A.25). The duration of chemical exposure had a significant effect on *in vitro* regeneration (Table 3.1; Table A.25). Nodal sections exposed to an anti-mitotic agent for only one day had significantly more plantlets (4.7) than either a three (2.0) or six day exposure (0.6) (Table 3.1). There was no significant effect of concentration on plantlet number, but like *M. punctata*, higher plantlet numbers were observed at the lower concentrations.

3.3.2 Effect of Duration, Chemical and Concentration on Vigor and Polyploidization

M. punctata has an average DNA content of 1.45pg in the diploid state or 2.87pg when in a tetraploid state (Figure 3.1 A, C, E); while *M. fistulosa* has DNA contents of 1.95pg and 3.81pg for the diploid and tetraploid state, respectively (Figure 3.1 B, D, F).

Polyploids were attained across a wide range of treatment combinations in both species and the success of *in vitro* chromosome duplication was greater than anticipated. For that reason a successful treatment needed further definition beyond simply producing tetraploid plants. Therefore, when determining the more successful treatments the total plantlets *in vitro* and in the greenhouse were also considered in addition to the total number of polyploids that were produced.

Table 3.1. Effect of concentration, duration and chemical on the total number of plantlets produced per petri dish (four node sections) *in vitro* by *M. punctata* and *M. fistulosa* after 16 weeks.

Oryzalin and Trifluralin concentration (µM)	Number of plantlets	
	M. punctata [z]	*M. fistulosa*
0	4.5*ab*	3.4*a*
1	5.3*a*	2.5*a*
5	3.6*ab*	2.9*a*
15	3.2*ab*	1.9*a*
30	2.5*ab*	1.3*a*
60	1.4*b*	1.1*a*
90	1.5*b*	1.3*a*
120	2.5*ab*	2.6*a*
Chemical		
Oryzalin	2.3*b*	1.9*a*
Trifluralin	3.5*a*	2.2*a*
Duration		
1	4.7*a*	4.7*a*
3	2.8*ab*	2.0*b*
6	1.6*b*	0.6*c*

[z] ***a-b***. Treatment means following the same letter in the same treatment and column are not significantly different (α=0.05) based on a Tukey's multiple range test.

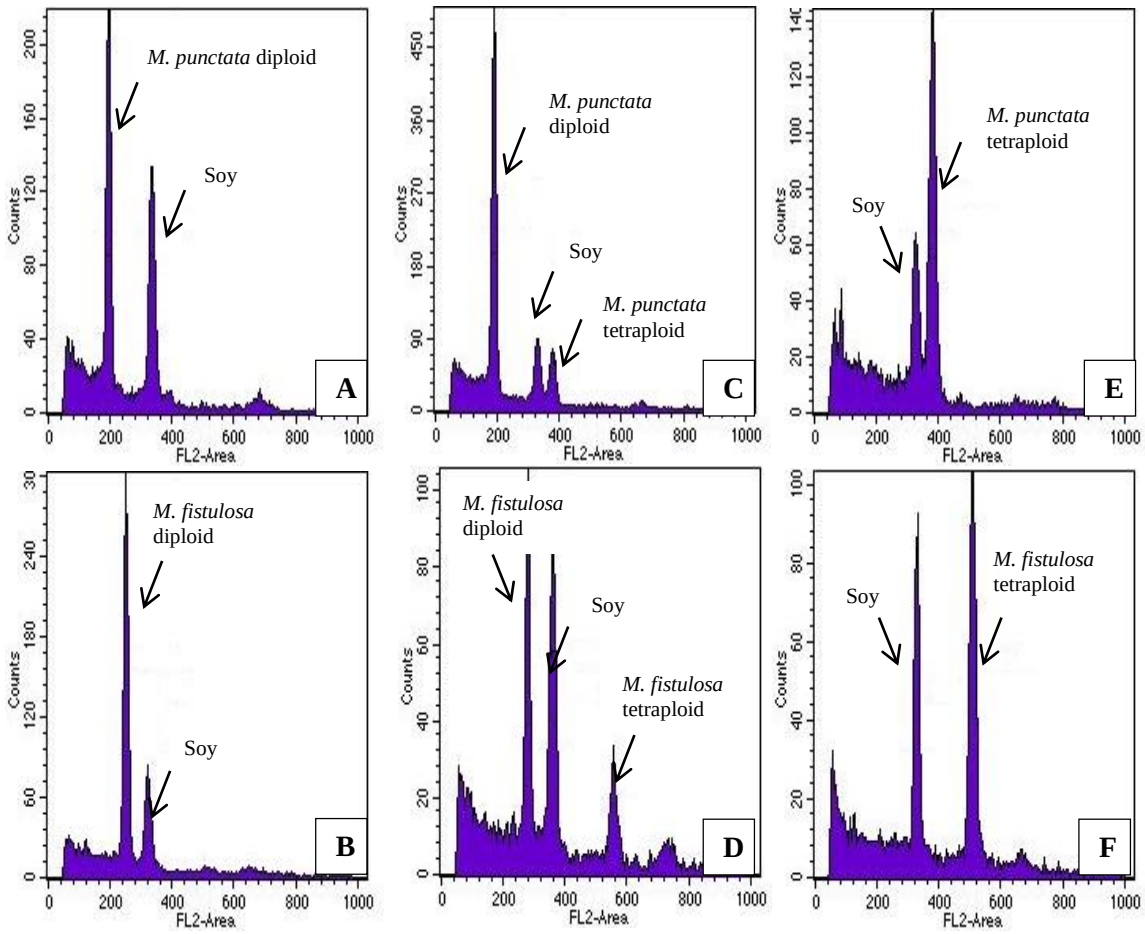

Figure 3.1. Example output from flow cytometery of diploid (A&B), mixoploid (C&D), and tetraploid (E&F) plants of *M. punctata* (A, C, E) and *M. fistulosa* (B, D, F).

Explants of *M. punctata* which were exposed to an anti-mitotic agent for one day produced the highest number of *in vitro* plantlets (606) but the lowest percentage of tetraploids (14%) which was balanced by the six day treatment producing the fewest number of *in vitro* plantlets (237) and the highest percentage of polyploids (35%) (Table 3.2; Figure 3.2). When just examining the percentage of polyploids produced it would appear that a six day exposure yielded the greatest proportion of tetraploids, however all three durations of exposure produced similar numbers of tetraploids (14, 14 and 15, respectively) (Table 3.2; Figure 3.2). Differences were observed in the number of plantlets produced and the number that were transferred to the greenhouse, meaning a balance between the number of tetraploids found and the total number of plantlets available to screen must be considered (Figure 3.2).

There were also differences observed in the response of *M. punctata* to the two chemicals applied. More *in vitro* plantlets were produced by nodal sections exposed to trifluralin than oryzalin (715 vs 543) as well as more plants in the greenhouse (124 vs 71). While the total number of tetraploids (i.e. across all concentrations) produced by oryzalin (19) was comparable to total number produced by trifluralin (24), oryzalin had a higher rate of polyploidization (27%) than trifluralin (19%) (Table 3.4).

When determining the effect of concentration on the outcome of polyploidization, there were minor differences between the two chemicals once again. Explants that were treated with 30µM oryzalin had the second greatest number of tetraploids (5) and highest percentage of polyploidization (50%) in contrast to the 60µM treatment which also produced a polyploidization rate of 50%, but only one plantlet out of the two tested was a tetraploid (Table 3.4).

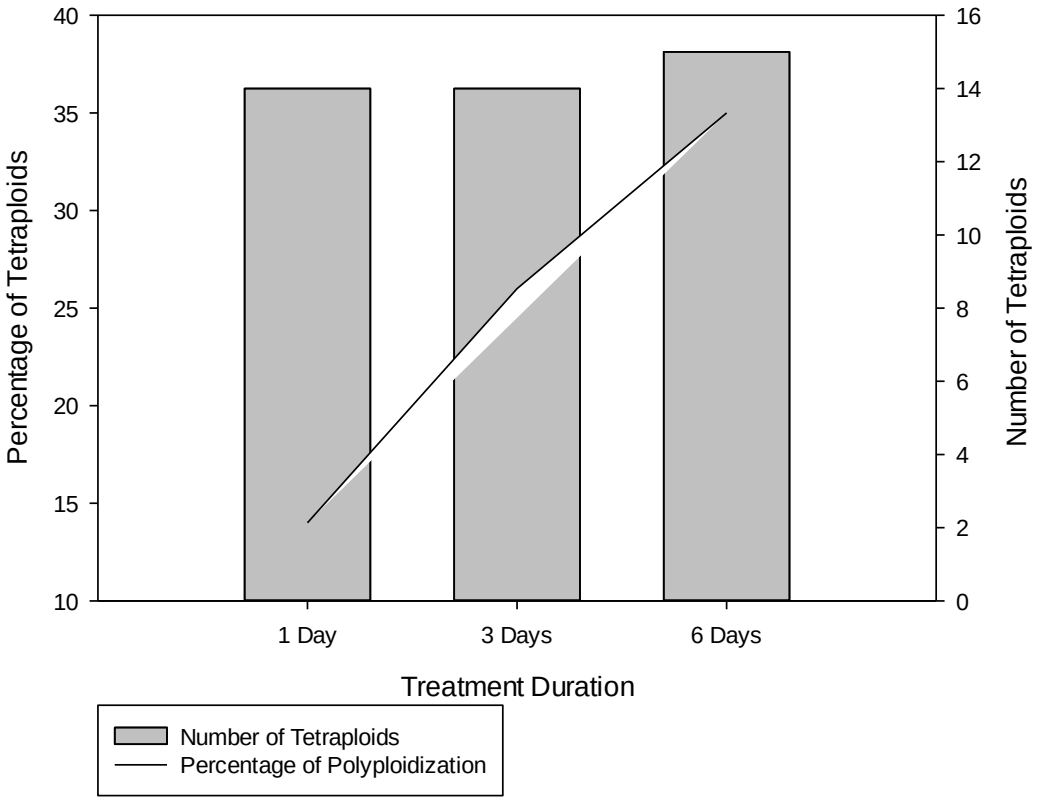

Figure 3.2. Total number of tetraploid *M. punctata* plants and percentage of tetraploid plants identified using flow cytometry when exposed to an anti-mitotic agent for one, three or six days.

Table 3.2. Total number of *M. punctata* shoots produced *in vitro* across all blocks, acclimatized plantlets and the number and percentage of tetraploids produced by *in vitro* treatments of seven concentrations of two anti-mitotic agents for three durations.

	in vitro shoots (*n*)	Plantlets in Greenhouse (*n*)	Tetraploids	
			(*n*)	%
Oryzalin				
1	131	21	4	19
5	52	7	1	14
15	59	16	6	38
30	75	10	5	50
60	64	2	1	50
90	53	2	0	0
120	109	13	2	15
Total	543	71	19	27
Trifluralin				
1	122	24	0	0
5	193	26	5	19
15	94	18	4	22
30	70	14	1	7
60	62	8	6	75
90	72	18	5	28
120	102	16	3	19
Total	715	124	24	19
1 Day	606	99	14	14
3 Days	415	53	14	26
6 Days	237	43	15	35

The highest number of tetraploids (6) produced in response to oryzalin was exposure to 15µM (38%) (Table 3.2). For trifluralin, there was a wider range of concentrations tolerated by the plantlets. Explants treated with trifluralin had a 75% ploypoidization rate when exposed to 60µM, across all three durations of exposure which produced a total of 6 tetraploids (Table 3.2). Treatments of 90µM and 5µM both resulted in 5 tetraploids and a percentage of 28% and 19%, respectively (Table 3.2).

M. fistulosa nodal sections treated for one day with an anti-mitotic agent had the greatest number of *in vitro plantlets* (609) but the fewest tetraploids (36%), this was followed by a three day exposure that produced fewer plantlets (321) but an increased proportion were tetraploids (47%). The six day treatment produced even fewer plantlets (114) and the highest proportion of tetraploids (64%) (Table 3.3; Figure 3.3). Based on actual numbers of polyploids produced, a one day exposure produced the greatest number of tetraploids (71) and six days the least (17) (Table 3.3; Figure 3.3).

Unlike *M. punctata, M. fistulosa* did not appear to be as sensitive to the anti-mitotic agent chosen for treatment. Both oryzalin and trifluralin treatments produced similar numbers of plantlets (475 and 569, respectively) and polyploids (57 and 67, respectively) (Table 3.3). When oryzalin was used in increasing concentrations, there was a varied but corresponding increase in the number of tetraploids identified. Nodal sections exposed to 120µM of oryzalin produced 18 tetraploids (62%), while a 15µM exposure resulted in 8 tetraploids (89%) (Table 3.3). When trifluralin was selected as the anti-mitotic agent, *M. fistulosa* produced tetraploid over a wider range of concentrations. Treatments of 30, 60, 90 and 120µM yielded 10 (36%), 9 (75%), 19 (68%) and 16 (67%) tetraploids, respectively.

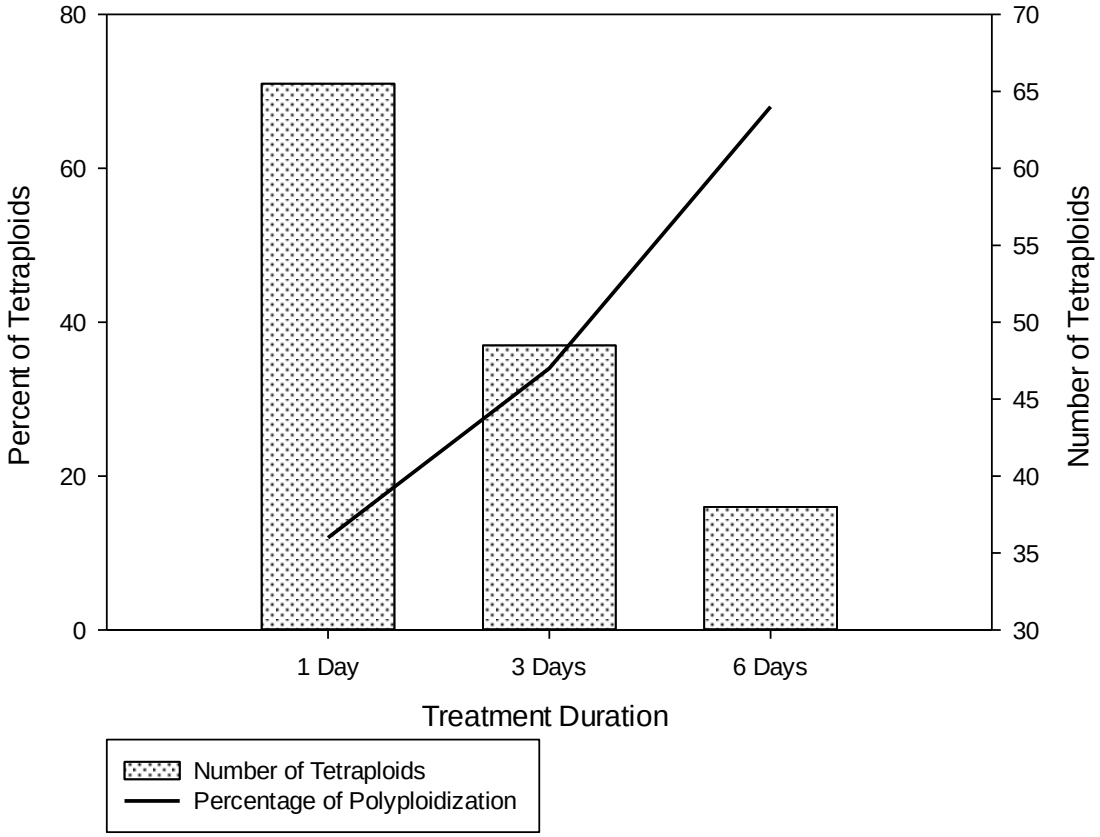

Figure 3.3. Total number of tetraploid *M. fistulosa* plants and percentage of tetraploid plants identified using flow cytometry when exposed to an anti-mitotic agent for one, three or six days.

Table 3.3. Total number of *M. fistulosa* shoots produced *in vitro* across all blocks, acclimatized plantlets and the number and percentage of tetraploids produced by *in vitro* treatments of seven concentrations of two anti-mitotic agents for three durations.

	in vitro shoots (*n*)	Plantlets in Greenhouse (*n*)	Tetraploids	
			(*n*)	%
Oryzalin				
1	77	35	9	26
5	116	23	5	22
15	57	9	8	89
30	30	10	7	70
60	45	22	3	14
90	34	15	7	47
120	116	29	18	62
Total	475	143	57	40
Trifluralin				
1	123	30	0	0
5	85	21	5	24
15	83	15	8	53
30	66	28	10	36
60	32	12	9	75
90	69	28	19	68
120	111	24	16	67
Total	569	158	67	42
1 Day	609	198	71	36
3 Days	321	78	37	47
6 Days	114	25	16	64

3.3.3 Correlation of Stomatal Length and Ploidy Level

For both species of *Monarda*, stomatal length was significantly correlated (P=<.0001) with ploidy level (Table A.26 and A.27). *M. punctata* had a moderate correlation of 0.60 between stomatal length and ploidy level. Between the lengths of 20 and 24µm there are approximately equal numbers of diploids and tetraploids, but at stomatal lengths equal to or greater than 25µm only one diploid was found (Figure 3.4). *M. fistulosa* had a slightly higher correlation of 0.72. When examining the distribution of stomatal lengths and ploidy of *M. fistulosa* two overlapping distributions are visible. The tetraploid and diploid distributions overlap between 16 and 20µm, and tetraploids become distinct from diploids at stomatal lengths greater than 21µm (Figure 3.5).

3.4 Discussion:

3.4.1 Effect of Chemical, Concentration and Duration on *in vitro* Regeneration

The anti-mitotic chemical and the concentration used had a significant impact on the total number of plantlets produced *in vitro* by *M. punctata*, but not by *M. fistulosa*; however, both species were significantly impacted by the duration of exposure. The negative effects of anti-mitotic chemicals applied at different concentrations, durations and their interactions have been reported for several species. There have also been reports documenting the interaction effect of the anti-mitotic chemical and the genotype.

Concentration and duration of exposure to anti-mitotic agents *in vitro* can negatively impact the ability of explants to regenerate. A study conducted in *Ranunculus* evaluated colchicine, trifluralin and oryzalin for the effectiveness of polyploidization as well as on the survival rate of unrooted plantlets (Dhooghe *et al.,* 2009). Higher concentrations of colchicine

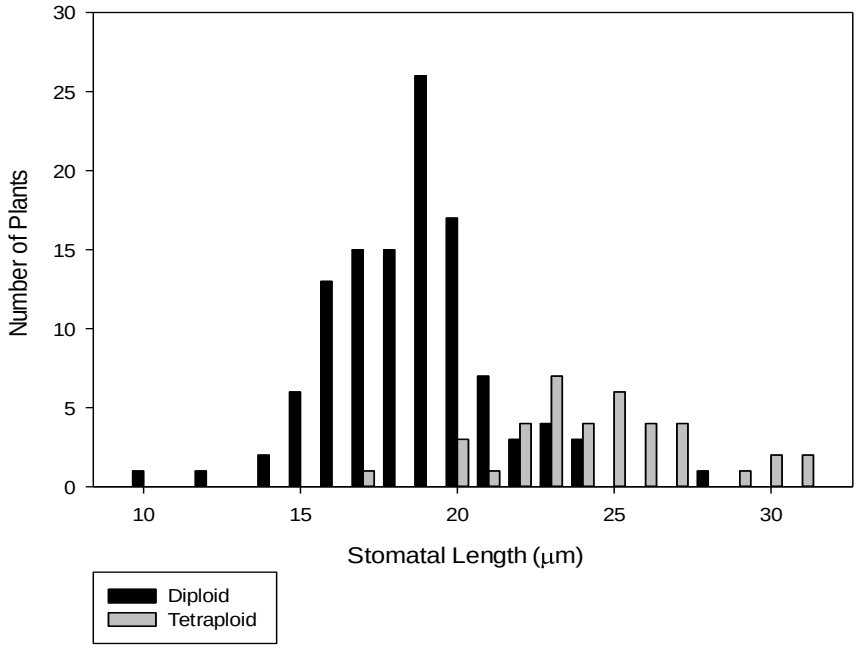

Figure 3.4. Distribution of the total number of confirmed *M. punctata* tetraploid or diploid plants across the measured average stomatal lengths.

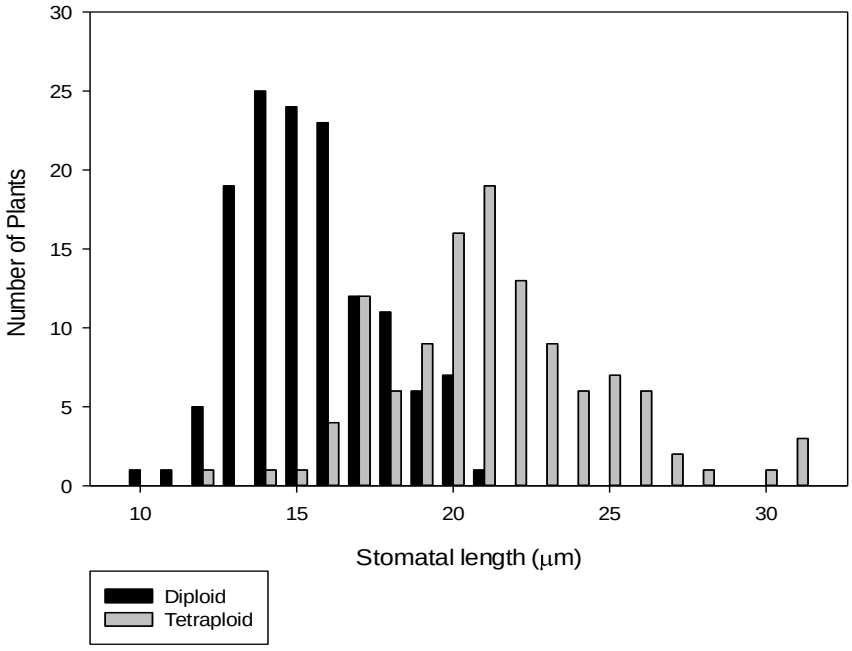

Figure 3.5. Distribution of the total number of confirmed *M. fistulosa* tetraploid or diploid plants across the measured average stomatal lengths.

did not impact the survival of the plantlets with control plantlets having a 89.3% survival rate and plantlets treated with 100µM or 200µM of colchicine having an 87.4% and 96.7% survival rate, respectfully (Dhooge *et al.,* 2009). The duration of exposure to colchicine also lacked any significant effect on survival. All concentrations of oryzalin significantly decreased the survival rate of plantlets, and an exposure greater than 4 weeks, also caused a significant decrease in survival (Dhooge *et al.,* 2009). Trifluralin had a significant impact on regeneration only when applied at the highest concentration (10µM), but no significant effect of duration was observed (Dhooge *et al.,* 2009).

A similar study using microshoots of six clones of Japanese quince (*Chaenomeles japonica*) examined the effect of oryzalin and colchicine when applied at six concentrations on the survival and number of polyploids produced *in vitro*. Colchicine decreased regeneration at increasing concentrations; however the severity of the effects were highly dependent on the specific clone used (Stanys *et al.,* 2006). For instance, clone 47 had a 98% survival when exposed to 250µM of colchicine which decreased to 50.4% when exposed to 38000µM of colchicine (Stanys *et al.,* 2006). In contrast, clone 43 had a 92% survival as 250µM of colchicine and only 6.7% survival at 38000µM colchicine (Stanys *et al.,* 2006). In their report, oryzalin had a higher *in vitro* survival. When exposed to the lowest concentration of oryzalin (10µM) clones 47 and 43 had 84% and 96% survival, respectively and at the highest concentration (144µM) the percentage of survival was still 90% and 96%, respectively (Stanys *et al.,* 2006). This study in Japanese quince (Stanys *et al.,* 2006) found a connection between the individual genotype of a species and the effect of the treatment. This is similar to the results of the current study where effect of species and genotype from a heterogenous population affected response to the chemicals.

3.4.2 Effect of Duration, Chemical and Concentration on Vigor and Polyploidization

In the current investigation, tetraploid plants of *M. punctata* and *M. fistulosa* were produced using a wide range of concentrations and durations of exposure of two different anti-mitotic agents. Duration of exposure did not affect the number of tetraploids produced by *M. punctata*; however the vigor did decrease as the length of exposure increased. This was demonstrated by the decrease in the total number of *in vitro* plants and the number of plants available for transfer into the greenhouse as the duration of exposure increased. These decreases resulted in a reduction in the total numbers of plants available to test and an increased percentage of polyploidization because the actual number of tetraploid plants were similar across all exposures. While the number of tetraploids produced by *M. punctata* was not affected by the duration of exposure, it was affected by the chemical and concentration.

Several investigations have been completed in a variety of species to determine the optimal protocol and the response of the species to treatments. Each of these studies have reported different results and different sensitivities for almost every plant studied. A study conducted using unrooted plantlets of *Ranunculus* reported optimal exposures and durations for oryzalin, trifluralin and colchicine (Dooghe *et al.*, 2009). They found that 16 or 24h exposure to 200µM colchicine resulted in 23% tetraploids and 2µM of trifluralin or 1µM oryzalin for 10 weeks had 28% and 19% tetraploidy, respectively (Dooghe *et al.*, 2009). Varying sensitivities to the chemicals were also reported (Dooghe *et al.*, 2009). At higher concentrations of colchicine, survival did not decrease and when exposed to trifluralin only the highest concentration tested (10µM) decreased survival rates (Dooghe *et al.*, 2009). However, oryzalin largely impacted the survival of the unrooted plantlets with a decrease of close to 30% from the lowest (0.5µM) to the

second lowest (1µM) concentrations tested. At the highest concentration of oryzalin (3µM), no plantlets survived the treatment (Dooghe *et al.,* 2009).

A study in Japanese quince (*Chaenomeles japonica*) explored the effect of species and genotype by applying a range of concentrations of oryzalin and colchicine to microshoots of different clones (Stanys *et al.,* 2005). They found that the rate of polyploidization was influenced less by colchicine than oryzalin and polyploids were recovered from all tested concentrations of both chemicals (Stanys *et al.,* 2005). However, the six clones responded differently to the concentrations of colchicine tested. Clones 93018, C19 and 18(9336) showed optimal tetraploid production when exposed to the lower concentrations of colchicine (1250µM-2500µM), compared to clones 43, 47 and 9365 that produced more tetraploids at higher concentrations (15,000µM-22,500µM) (Stanys *et al.,* 2005). An effect of species and genotype was also demonstrated for oryzalin. A concentration of 30-40µM was determined to be optimal exposure and at 30µM of oryzalin, clones 43 and 9365 had a polyploidization rate of 17% and 11%, respectively, while clones 47 and 18 only had 3% and 9% polyploidization (Stanys *et al.,* 2005).

Another study in three *Rosa* species examined the effect of three anti-mitotic inhibitors applied to nodal sections *in vitro* (Khosravi *et al.,* 2003). This study used oryzalin, trifluralin and amiprophosmethyl (APM) to induce polyploidization across five concentrations (0, 3, 6, 12 and 24µM) and three durations of exposure (12, 24 and 48h) (Khosravi *et al.,* 2003). There were no differences observed between the ability of the three chemical's to induce tetraploidy at a concentration of 6µM for 24h (Khosravi *et al.,* 2003). However, species and cultivars did respond differently. Oryzalin at 6µM and 24h exposure resulted in 60%, 6.3% and 0% tetraploidy in *R. persica*, *R. hybrida* cv. Iceburg and *R. hybrida* cv. Akito, respectively (Khosravi *et al.,* 2003). There was also an inverse relationship between the percentage of tetraploids and the

survival of the treated nodal sections (Khosravi *et al.*, 2003). When treated with 24µM of either APM or trifluralin for 48h the highest percentages of polyploidization were achieved (68% and 63%, respectively), however these were accompanied by the lowest percentages of survival (30% and 40%, respectively) (Khosravi *et al.*, 2003). These results are similar in *Monarda*. Both species of *Monarda* showed different responses to the treatment applied. *M. fistulosa* was more responsive to polyploidization than *M. punctata* with better survival and higher numbers of tetraploids.

While the response of the two *Monarda* species to the treatments for polyploidization varied, this variation is similar to what has been reported in other species. Often, combinations of chemicals, durations, or concentrations are reported and vary in effectiveness amongst species and genotypes. The initial genotypes of both *Monarda* species used in this study were selected from a heterogeneous population. The effect of plant to plant genotype variability on the regeneration of nodal sections *in vitro* was demonstrated in Chapter 2. This background variation may also affect the ability of the anti-mitotic agents to induce chromosome duplication. However, while variability within the population of *Monarda* may initially be seen as an obstacle to optimize protocols, this variation provides a more robust method for breeding and is inherent in native species.

3.4.3 Correlation of Stomatal Length and Ploidy Level

There are several indicators other than nuclear DNA content which have been shown to be effective in identifying potential polyploids. Nuclear DNA is the most definitive protocol for ploidy determination; however it can also be time consuming, costly and requires large and expensive equipment. Using other methods for pre-screening can quickly reduce the size of the

population which needs to be verified through flow cytometry. Other indicators which have been used to verify ploidy levels include: guard cell and chloroplast number, pollen grain diameter and stomatal length (Cohen and Yao, 1996).

Studies in *Zantedschia* cultivars (Cohen and Yao, 1996) as well as in Japanese quince (Stanys *et al.*, 2006) both successfully used the length of the stomatal pores as an indicator of ploidy. Stanys *et al.*, (2006) reported an increase in stomatal length of approximately 33% between a diploid and the corresponding tetraploid clone. Cohen and Yao (1996) also found significant differences between the stomatal lengths, with ploidy accounting for 78% of all variation between stomatal measurements. They also tested the use of stomatal lengths as a pre-screening method and from a subsample of 110 plants, they were able to select 40 with 91% accuracy (Cohen and Yao, 1996). Clearly the use of a rapid and inexpensive pre-screen tool has many advantages and both *M. punctata* and *M. fistulosa* showed a good correlations between ploidy and stomatal length which would facilitate the use of stomatal length as a screening tool with relative accuracy.

There are several morphological characteristics which have been associated with an increase in ploidy level. Some phenotypic characters include increased leaf size as well as darker, thicker leaves. However other characteristics such as an increase in stomatal length have been used successfully in other species in order to help identify tentative polyploids. Being able to identify polyploids through morphological assessments is useful as it reduces the number of samples to measure using expensive equipment such as a flow cytometer and can be used when such equipment is not available.

3.5 Summary:

This study resulted in an efficient method to produce tetraploids in *Monarda punctata* and *M. fistulosa* by exposing nodal explants to a range of oryzalin and trifluralin treatments. The number of tetraploids produced was sufficient to use in further analysis and a breeding program and could help generate new lines for the ornamental market. The correlations between stomatal length and ploidy level provide a good tool to efficiently screen for polyploids. The protocols developed for flow cytometry provided definitive identification of the polyploids. Overall, this study allowed for the rapid induction and identification of tetraploid *Monarda* which will be used in further investigations.

4.0 Chapter 4: The Effect of Ploidy Level on Total Phenolic Content and Antioxidant Properties of *Monarda punctata* and *M. fistulosa*

Abstract:

Genetically related tetraploid, diploid and chimeric leaf extracts of ten genotypes of *M. punctata* and nine genotypes of *M. fistulosa* were tested for their total phenolic content (TPC) and percentage antioxidant activity (%AA) using Folin-Ciocalteau and Diphenylpicrylhydrazyl (DPPH) assays. The TPC of four genotypes of *M. punctata* were significantly increased with an increase in ploidy level, and these same genotypes subsequently had four of the five lowest diploid TPC. However chromosome duplication did not have an effect on their %AA. *M. fistulosa* had a similar response for both the changes in TPC and %AA. Four genotypes had a significant decrease in TPC and %AA with an increase in ploidy level with a fifth genotype having a significant decrease in %AA. In contrast to the general trend, there was one genotype of *M. fistulosa* which had a significant increase in both %AA and TPC, while a second genotype had an increase in TPC. These results indicate that both genotype and ploidy level can influence the TPC and %AA of leaf extracts both positively and negatively. It also indicated a correlation between initial content and influence of chromosome duplication as well as supporting the link between TPC and %AA.

4.1 Introduction:

M. fistulosa and *M. punctata* are members of the family Lamiaceae; other members of this family include: basil, mint and coleus. While these two species are a part of the same genus, they belong to different sub-genera; *M. fistulosa* is a member of the sub-genus *Fistulosa* while *M. punctata* belongs to *Cheilyctis* (Scora, 1967a). This classification is partially a result of the different chromosome numbers of the species. Members of *Fistulosa* are reported to have a

chromosome number of 2n=2x=36, while *Cheilyctis* has 2n=2x=22 chromosomes (Scora, 1967a). Both species are classified as native to Ontario and are commonly known as wild bergamot (*M. fistulosa*) and spotted bee balm (*M. punctata*) (Anderson, 2000 & 2003). Members of the genus are widely spread across North America and in most places where this genus is found it has gained some economic importance either as an ornamental plant, as a source of food and flavouring, or a medicinal treatments (Scora, 1967b).

Both *M. punctata* and *M. fistulosa* have been the subject of studies investigating their essential oils, components and medicinal effects. The oils of both *M. punctata* and *M. fistulosa* have potential uses as a lipase inhibitor (*M. punctata*) (Yamada *et al.,* 2010), anti-inflammatory remedy (Zhilyakova *et al.,* 2009) and for the treatment of several varieties of cancer (*M. fistulosa*) (Johnson *et al.,* 2012).

A study which was first completed with 80% acetone whole plant extracts of *M. punctata* and *in vitro* mouse plasma revealed that a major constituent of this oil (i.e. carvacrol) demonstrated a concentration-dependent inhibition of inflammation with an IC_{50} value of 4.07mM (Yamada *et al.,* 2010). Upon further investigation, it was found that carvacrol isolated from *M. punctata* essential oil significantly decreased swelling in mice paws when compared to a control group (Yamada *et al.,* 2010). Another study carried out in *M. fistulosa* was investigated the effects of two essential oil components on several human carcinoma cell lines (Johnson *et al.,* 2012). Thymoquinone and thymol were isolated from *M. fistulosa* essential oil as two of the major components and thymoquinone showed high affinities for the cell lines (Johnston *et al.,* 2012). Specifically, it was selective for the human prostate carcinoma cell line with an ED_{50} value comparable to that of a common chemotherapy drug (Johnston *et al.,* 2012). The potential of these oils is just beginning to be explored, and it is promising. A relatively fast and efficient

method of producing more oils, with increased medicinal activities and compounds could further the potential of these two species.

Induced autotetraploidy is a means to induce genome duplication and create new progeny with a duplicate set of chromosomes. Total phenolic and antioxidant activity testing completed using autotetraploid leaf extracts has led to interesting reports. Firstly, there have been documented cases of increased production of essential oils (Janaki-Ammal and Gupta, 1996) as well as accumulation of secondary metabolites (Lavania *et al.,* 2012) in tetraploid plants as compared to their diploid progenitors. Another potential benefit of chromosome duplication for essential oil production is the possibility of an altered chemical profile. There have been documented cases in which particular oil constituents that were present in the diploid were no longer present in tetraploid oil and instead new compounds are found (Murray and Williams, 1976).

Total antioxidant and phenolic contents are a common measure for both leaf extracts and essential oils in order to gauge the medicinal potential of the components. Total antioxidant activity can be assessed using several assays, however a commonly used method is DPPH. This method measures the scavenging ability of the oil/extract to bind and neutralize the free radical 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{+}$) compared to the standard Trolox (water soluble vitamin E) (Javanmardi *et al.,* 2003). The reaction results in a colourmetric change as the radicals are neutralized and sample absorbance's are compared. The calculation of total phenolic content (TPC) is completed with a similar assay known as the Folin-Ciocalteau assay. As with the DPPH assay, samples are compared to a gallic acid equivalent in a colourmetric reaction which is measuring the transfer of electrons from phenolic compounds to acid complexes (Ainsworth and Gillespie, 2007).

Leaf extracts are commonly used in place of essential oils for testing in assays such as Folin-Ciocalteau and DPPH as leaf extracts can be done much quicker, with less plant material, labour and lower cost. A comparative study completed in a member of the Lamiaceae family, thyme (*Thymus vulgaris* L.) investigated the results of three phytochemical assays on twelve accessions of essential oils and leaf extracts (Chizzola *et al.*, 2008). Two solvents were used in the preparation of the leaf extractions, varying percentages of ethanol and dichloromethane (CH_2Cl_2) (Chizzola *et al.*, 2008). The composition of the essential oils when examined using GC-MS was very similar to that found in the CH_2Cl_2 leaf extracts (Chizzola *et al.*, 2008).

Previously, diploid and tetraploid cytotypes of *M. punctata* and *M. fistulosa* were attained through the application of oryzalin and trifluralin *in vitro* (Chapter 3). The cytotypes used for this study were derived from nodal sections of the same explant donor *in vitro* and were classified as diploid or tetraploid by means of stomatal length measurements and flow cytometry. The objective of this study is to determine the effect of ploidy level on the total phenolic content and total antioxidant activity of genotypically paired diploid, tetraploid and chimeric leaf extracts of *M. punctata* and *M. fistulosa*.

4.2 Materials and Methods:

4.2.1 Leaf Extractions

Approximately 0.5 g of leaf material was taken from the upper 4-5 nodes, placed in a 15mL polypropylene centrifuge tube (Fisher Scientific, Canada) and frozen in liquid nitrogen. Three samples were taken from every plant sampled and were stored in a -80°C freezer until freeze dried overnight (Freezeone 4.5; Labconco, Kansas City, USA). Dried leaf matter was then

weighed again and placed into a 50 mL centrifuge tube with two grinding beads. Samples were pulverized to a fine powder using the Geno/Grinder (Spex Sample Preperation, USA).

Small subsamples of leaf powder were weighed using an analytical balance. Ideally a minimum weight of 0.05g was attained and placed into a 1.5mL centrifuge tube. However, there where instances in which this weight could to be achieved and then the maximum possible was taken.

Methanol (MeOH) (80%) was added to the centrifuge tubes in a ratio of 1mg tissue :10µL solvent and all samples were placed in a floating rack and sonicated for 3 hours using a sonicating bath (Bransonic 3510R-DTH, 42 KHZ). Once sonication was completed, tubes were placed into a Thermo Sorvall Legend Micro 21 (Thermo Fisher Scientific, Bremen, Germany) and centrifuged for 10 minutes at 14,800 rpm. The supernatant was removed and placed into a clean 1.5mL centrifuge tube, and the pellet discarded. Samples were stored in a -20°C freezer until further use.

The genotypes chosen were created and verified by induction of polyploidy described in Chapter 3. Briefly oryzalin and trifluralin were used to induce autotetraploids in diploid *M. punctata* and *M. fistulosa.* The ten pairs of genotypes consisted of a diploid and its derived tetraploid (Table B.4). This allowed for the direct comparison of the effect of increased ploidy level on %AA and TPC.

4.2.2 Folin-Ciocalteau Assay

Fresh gallic acid (GA) standard was made each day the assay was run. GA (0.05g) (Sigma Aldrich, Oakville, Ontario) was dissolved in 500µL of 100% MeOH and the final volume was brought to 5mL with water. A dilution series was completed with distilled water to

final concentrations of: 1000, 500, 250, 125, 62.5 and 31.25 µg/mL. Folin-Ciocalteau reagent (FCR) (MP Biomedicals, OH, USA) was then placed into a 60mL centrifuge tube in a dilution of 1:10 FCR:distilled water.

A GA dilution series was pipetted (10µL) into a 96 well clear flat bottom plate in triplicate using a multichannel pipette (Gibson P200 pipetman neo). Distilled water and 80% MeOH were pipetted into the plate in the same manner. Twelve leaf extract samples were run in each plate. 10µL of these samples were pipetted into four wells on the plate. A volume of180µL of distilled water was added to the last row of all samples and which were used as the sample blanks. All of the remaining samples had an additional 100µL of FCR added and after 5 minutes 80µL of 0.25M Na_2CO_3 was added to the wells in the same order as the FCR. The plate was then incubated in the dark at room temperature for 1 hour. Once the reactions were complete, the absorbances were measured at a wavelength of 725 nm using a microplate reader (BioTek Synergy H1 Hybrid Reader). One extract was run in triplicate on a single plate and a second leaf extract derived from the same plant source was run, also in triplicate, on a second 96 well plate.

4.2.3 DPPH Assay

A 1mM trolox standard stock solution was made at the beginning of each day the assay was run. Trolox (Sigma Aldrich, Oakville, Ontario) was weighed out using an analytical scale (Sartorius CP124S) and dissolved in 80% MeOH. A serial dilution was then made to a final concentration of 500, 250, 125, 62.5 and 32.125 µM. A fresh DPPH (Sigma Aldrich, Oakville, Ontario) solution was made at the beginning of each day required to a final concentration of 150µM by dissolving the powder in 80% MeOH.

Much the same as the Folin-Ciocalteau assay, a 96 well clear flat bottom plate was used and 25µL of all samples were pipetted into the plate in triplicate using a multichannel pipette. Blanks of both the MeOH and distilled water were also included as controls. A volume of 200µL of DPPH was added to all wells expect those containing sample blanks, which instead received 200µL of 80% MeOH. The plates were then incubated in the dark at room temperature for 6 hours to allow the reactions to come to completion. The sample absorbances at 517nm were then obtained using a microplate reader (BioTek Synergy H1 Hybrid Reader)**.**

4.2.4 Calculation of Total Antioxidant Activity, Total Phenolics and Statistics

The total antioxidant activity (%AA) was calculated in percentage using the following equation: $\%AA = [(A_0-A_1)/A_0] \times 100$, where A_0 is the recorded absorbance of the control reaction (all reagents except extract) and A_1 is the measured absorbance of the leaf extract (Adbelhady *et al.*,2011).

For the calculation of TPC first, the average absorbance of the GA standards was calculated and plotted graphically to obtain a standard curve and a line of best fit (y=mx+b). The corrected absorbance for each of the three sample values was then calculated by subtracting the sample's blank and the MeOH absorbance values from the sample absorbance with the addition of FCR and Na_2CO_3 (cor abs = sample abs – sample blank – MeOH blank). The gallic acid equivalence (GAE) was then determined in µg/mL by solving for *x* using the line of best fit and the correct absorbance value as *y* (GAE = (cor abs – b) / m). GAE was thenback calculated to the plant material and expressed in $\mu g_{(GA)}/mg_{(tissue)}$. This was done by multiplying the GAE by the volume of MeOH added for extraction divided by the weight of the subsample of leaf powder used for the extraction (GAE µg/mg = GAE*(µL MeOH/mg leaf powder).

The GAE µg/mg tissue and %AA of all samples were analyzed using the Proc Mixed procedure and means were compared using a Tukey's pairwise comparison (SAS 9.3 Institute, Inc., Cary, NC). Each of the two plates were considered to be individual blocks and the three samples per plate were replicates.

4.3 Results:

4.3.1 Effect of Ploidy and Genotype on Total Phenolic Content of Leaf Extracts

The interaction of genotype and ploidy level was found to be significant, as well as the main effects. The interactive effect was examined and contrasting statements were made to examine the effect of ploidy on corresponding diploid and tetraploid pairs.

Both genotype and ploidy level (P=<.0001; Table A.26; Table A.28) were significant and this interaction significantly impacted the total TPC (P=<.0001; Table A.27; Table A.29) in the leaf extracts of *M. punctata* and *M. fistulosa*. In all but one genotype tested for *M. punctata* there was an increase in TPC observed in the tetraploid compared to the diploid (Figure 4.1). For four out of nine *M. punctata* genotypes (2, 6, 8 and 9) the differences were significant (P=0.0013; 0.0329; 0.0025 and <.0001, respectively; Table A.26) between the diploid and tetraploid cytotypes. Interestingly, the one instance in which chromosome duplication did not increase the TPC was in the genotype 7, which has the highest diploid TPC (Figure 4.1). This coincides with the observation that the four tetraploid genotypes which experienced a significant increase in TPC were derived from the plants with the lowest diploid TPC values (Table 4.1). The chimeras tested appeared to be variable in their TPC. For instance, the chimeric genotypes 1 and 3 had TPC which were lower than or equivalent to the diploid cytotypes, while those chimeras from genotypes 2, 5 and 10 had higher values that were closer to tetraploid values (Figure 4.1).

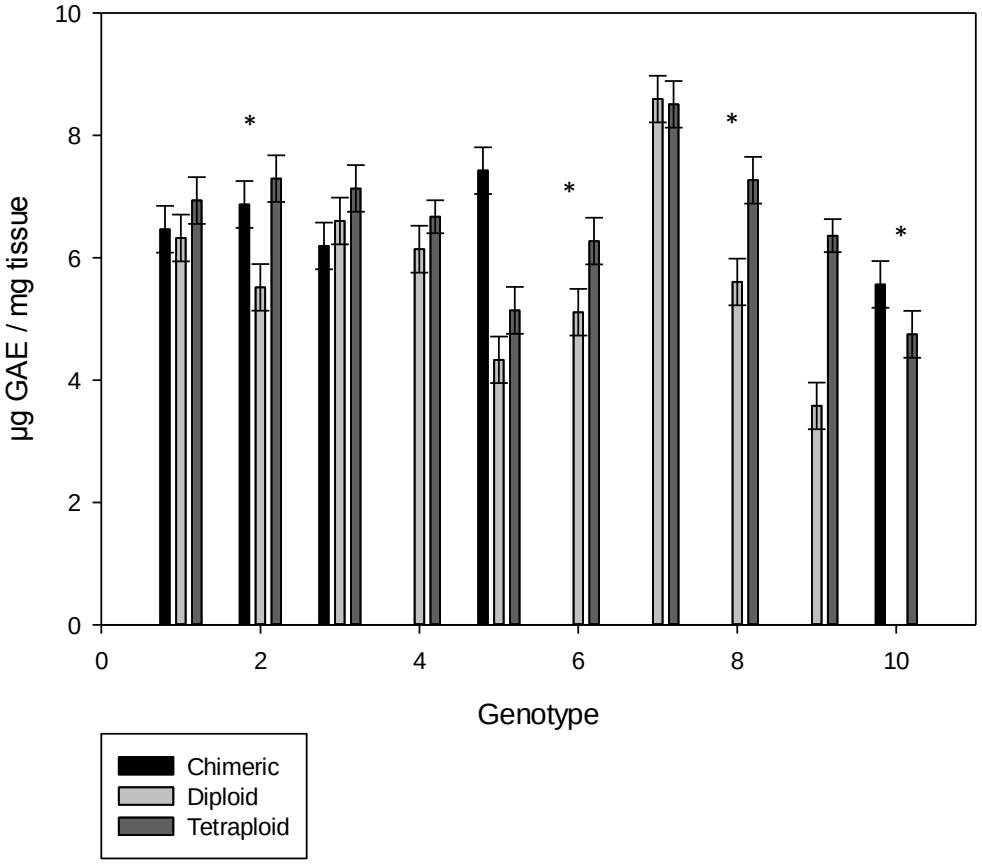

Figure 4.1. Total phenolic content (µg Gallic Acid Equivalents/mg tissue) of 10 diploid, tetraploid and chimeric *M. punctata* genotypes.
* denotes significant difference between diploid and tetraploid GAE (α=0.05)

Table 4.1. Total phenolic content (µg Gallic Acid Equivalents/mg tissue) of nine diploid *M. punctata* genotypes ranked from highest to lowest and the corresponding increase in GAE observed as a result of chromosome duplication.

Gentoype	Diploid GAE mg/mL	Percentage Increase
9	3.7 *g*	77.8
5	4.3 *fg*	18.7
6	5.1 *defg*	22.8
2	5.5 *bcdefg*	32.2
8	5.6 *bcdef*	29.7
4	6.1 *bcdef*	8.6
1	6.3 *bcdef*	9.7
3	6.6 *abcde*	8.0
7	8.6 *a*	-1.0

Six out of the eight genotypes of *M. fistulosa* tested were found to have significant differences in their total TPC as a result of genome duplication (Figure 4.2; Table A.28). Genotypes 2, 6, 8 and 9 had significantly lower TPC as a result of increasing ploidy (Figure 4.2; Table A.28). Only genotypes 1 and 7 had a significant increase in TPC after tetraploidization (Figure 4.2). While there appeared to be a relationship between the percentage increase in TPC after chromosome duplication for *M. punctata*, this relationship was not present for *M. fistulosa.* The three diploid genotypes with the lowest values all had positive but very different increases in TPC. However, in the middle to upper range, four genotypes all showed 30-36% decreases in TPC as a result of tetraploidization (Table 4.2).

4.3.2 Effect of Ploidy and Genotype on Total Antioxidant Activity of Leaf Extracts

The percentage antioxidant activity for the leaf extracts of *M. punctata* was significantly affected by genotype (P=<.0001; Table A.31) and the interaction of genotype and ploidy (P=<.0001; Table A.31). The leaf extracts of *M. punctata* also lacked any significant difference between the different cytotypes for each individual genotype (Figure 4.3). *M. fistulosa* leaf extracts for %AA were also significantly affected by ploidy (P=0.003; Table A.33) and genotype (P=<.0001; Table A.33), as well as the interaction of the two (P=<.0001; Table A.32). Unlike *M. punctata*, however, *M. fistulosa* did demonstrate differences between the diploids and tetraploids of the same genotype. Six out of the nine genotypes tested had significant differences observed, and in five out of those six there was a decrease in %AA of the tetraploid extracts as compared to the diploids. It should be noted that four of these five genotypes (2, 6, 8 and 9) which expressed a decrease in %AA were also the four genotypes which demonstrated a reduced TPC content in

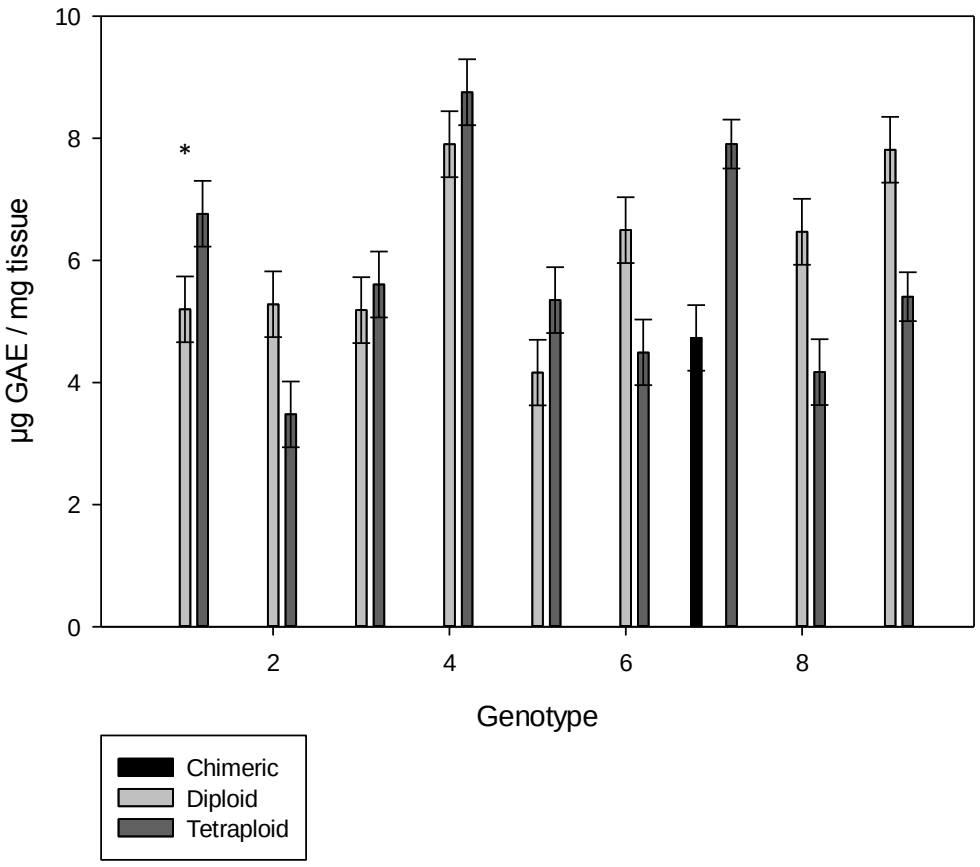

Figure 4.2. Total phenolic content (µg Gallic Acid Equivalents/mg tissue) of 10 diploid, tetraploid and chimeric *M. fistulosa* genotypes.
* denotes significant difference between diploid and tetraploid GAE (α=0.05)

Table 4.2. Total phenolic content (µg Gallic Acid Equivalents/mg tissue) of nine diploid *M. fistulosa* genotypes ranked from highest to lowest and the corresponding increase in GAE observed as a result of chromosome duplication.

Gentoype	Diploid GAE mg/mL	Percentage Change
5	4.2 *ef*	28.6
3	5.2 *def*	8.1
1	5.2 *def*	30.1
2	5.3 *cdef*	-34.2
8	6.5 *abcde*	-35.6
6	6.5 *abcde*	-30.8
9	7.8 *abc*	-30.8
4	7.9 *ab*	10.8

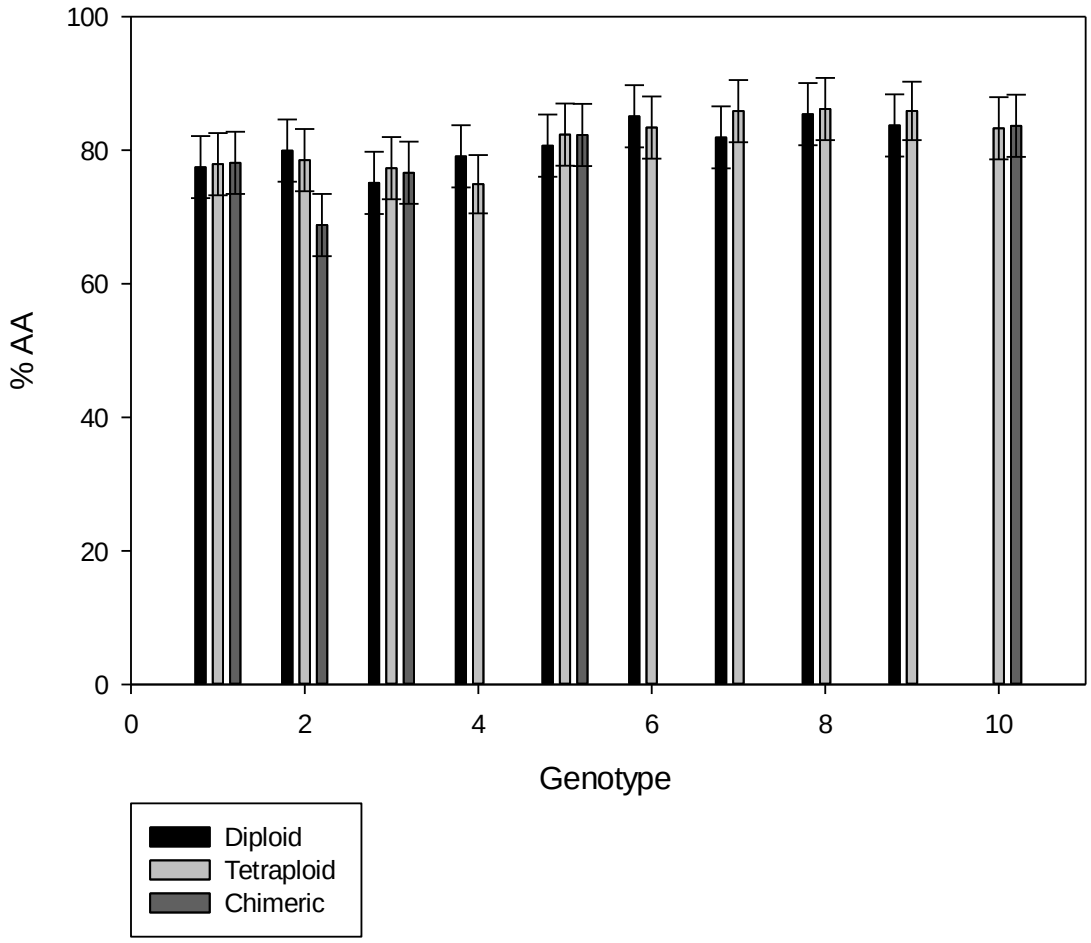

Figure 4.3. Percentage antioxidant activity of 10 diploid, tetraploid and chimeric *M. punctata* genotypes.

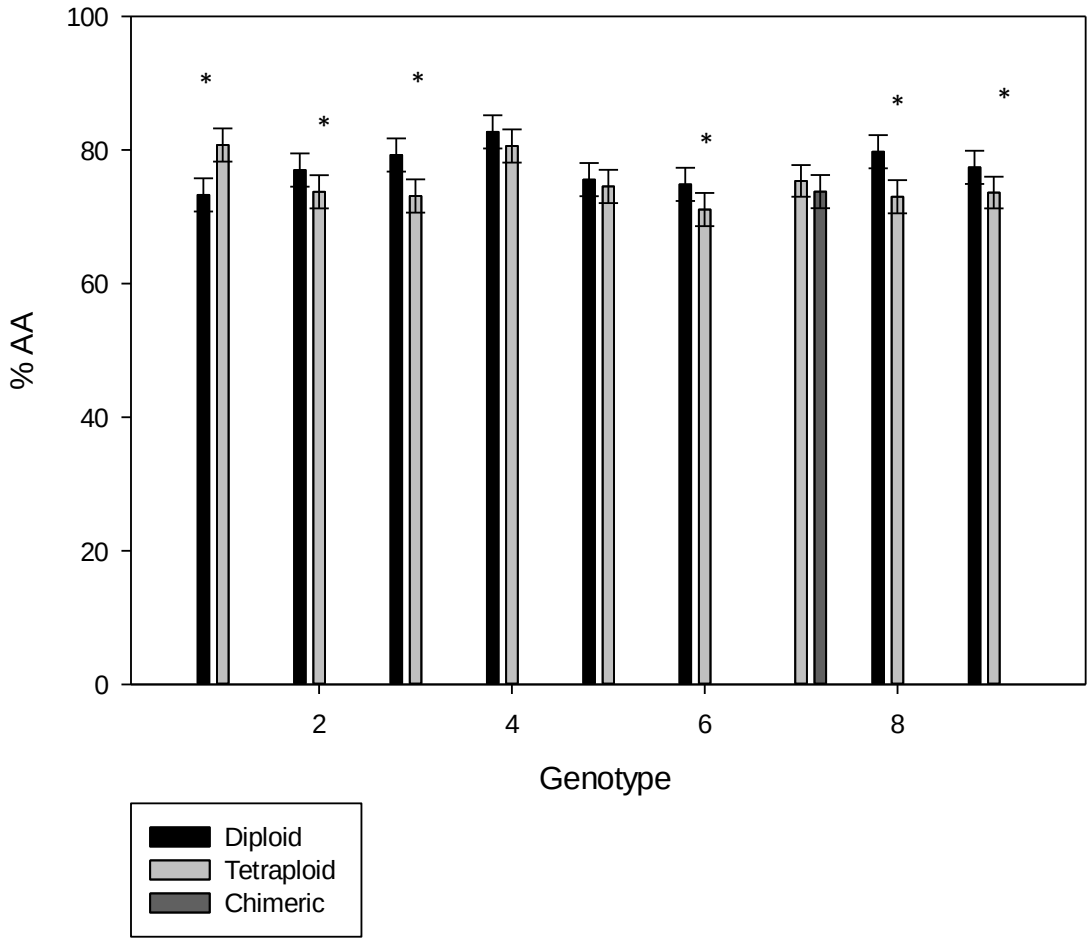

Figure 4.4. Percentage antioxidant activity of 10 diploid, tetraploid and chimeric *M. fistulosa* genotypes.
* denotes significant difference between diploid and tetraploid %AA (α=0.05)

the tetraploid cytotypes. The same is true for genotype 1 which experienced both a significant increase in %AA as well as TPC content from its diploid progenitor to the tetraploid. Genotype 7 which contained the only chimeric plant tested was not significantly different from its tetraploid version.

4.4 Discussion:

Both ploidy level and genotype are important factors in determining the total phenolic content and percentage antioxidant activity of *M. punctata* and *M. fistulosa*. The TPC of *M. punctata* had a positive response to chromosome duplication and resulted in a trend between diploid and tetraploid contents. Chromosome duplication however, did not have any effect on the %AA in the leaf extracts of *M. punctata*. One study completed in cytotypes of wheat (*Triticum spp.*) reported that on average the ancient diploid wheat varieties had half the TPC of durum or common wheat which has higher ploidy levels (Ciccoritti *et al.,* 2012). A similar study conducted in ginger (*Zingiber officinale* Roscoe) quantified the content of gingerol, TPC and the antioxidant activity in 18 diploid and tetraploid cultivars (Sanwal *et al.,* 2010). The tetraploid cultivars had a much higher content of gingerol compared to the diploid type, with up to a 29% increase observed, while no increase in TPC was observed (Sanwal *et al.,* 2010). The cause of this increased gingerol content is not quite clear, nor was it speculated upon. However, it may be possible that the second gene present in the tetraploids resulting in increased metabolic activity/gene expression thereby increasing the concentration of secondary metabolites (Lavania *et al.,* 2012).

Both species demonstrated significant variability between the genotypes as well as the cytotypes. This is an important consideration for the potential of selecting elite lines. For

instance, in some lines of *M. fistulosa* chromosome doubling could lower the phenolic content or antioxidant activity. Genotype has been reported to play a significant role in the chemical constituents of plants (Xie *et al.,* 2012). A study completed using different samples of diploid and tetraploid *Gynostemma pentaphyllum* found significant variability in the TPC of the samples (Xie *et al.,* 2012). For instance, a leaf from the diploid of genotype 3 had a TPC of 41.2 mg GAE/g while the diploid of genotype 4 only had a TPC of 22.5 mg GAE/g (Xie *et al.,* 2012). This was a similar issue when examining their tetraploid populations, the tetraploid from genotype 2 had a TPC of 14.8 mg GAE/g while genotype 4 only had a TPC of 8.1mg GAE/g (Xie *et al.,* 2012). A second study conducted in specialty potatoes reported significant effects of genotype on both TP and AA for each location and each year tested (Reddivari *et al.,* 2007).

M. fistulosa however, responded in the opposite manner as hypothesized. Seventy-five percent (6/8) of the genotypes tested resulted in significant differences in both TPC and %AA with 67% (4/6) and 83% (5/6) of those representing a decrease in activity from the diploid to the tetraploid cytotypes. The study conducted in *Gynostemma pentaphyllum* also suggested a relation between the TPC and the AA, as well as reporting higher TPC and scavenging activities for the diploid compared to tetraploid genotypes (Xie *et al.,* 2012). The relative DPPH radical scavenging capacity was also higher for the diploids than for the tetraploids (Xie *et al.,* 2012).

The possible reason that TPC and %AA may decrease with an increase in ploidy level is largely unknown, as is why these values increase. One study conducted in species of *Cymbopogon* found a correlation between oil components and response of body size to chromosome duplication (Lavania *et al.,* 2012). They reported that plants with essential oils rich in aldehydes respond to chromosome duplication by reducing growth while those with alcohol rich oils demonstrate an increase in vigor with an increase in chromosome number (Lavania *et*

al., 2012). The same study also noted an increase in size of essential oil secreting cells but a subsequent decrease in the number of these cells (Lavania *et al.*, 2012). It is possible that the varying responses of the two species tested are related to their different chromosome number, their oil components or a difference in biochemical responses to induced chromosome doubling.

These results are limited in the number of available genotypes to test as well as the general state of the material available at the time of extraction and testing. Plantlets for the leaf extraction were acclimatized to the greenhouse as they became large enough *in vitro* and as time and help was available. This resulted in plants being present in the greenhouse for different periods of time before material was harvested for extraction, although only the newest 4-5 leaves were harvested. The physical condition of the plants was also questionable during the harvesting of material. The plants had become largely infested and this may have altered their chemical profiles, although it is notable that all plants were in the same condition. The cleanest looking leaves were selected for extractions.

4.5 Summary:

The response of the total phenolic content and percentage antioxidant activity of *M. punctata* and *M. fistulosa* to genome duplication was highly variable. The %AA of *M. punctata* was mostly unaffected by genome duplication, while the TPC was positively influenced. All genotypes except one experienced an increase in TPC with an increase in ploidy, and in four of these which the measure was significant. There was a relationship between an initially low diploid TPC and corresponding increase in tetraploid TPC reported. The cytotypes of *M. fistulosa* had a similar response in both assays. Four of the same genotypes demonstrated a significant decrease in both TPC and %AA when tetraploidy was induced; however only one

genotype had a significant increase in both values after genome duplication. These results demonstrate the potential of chromosome duplication to increase phenolic and antioxidant activities of medicinal plants. Success was highly dependent on species and genotype. To increase that opportunity of success, it would be advisable to work with a large number of genotypes within a species. Further investigations which need to be completed include isolated and quantifying the components of the essential oils as well as quantifying the distilled essential oil volumes of both cytotypes on a per gram basis.

5.0 Chapter 5: Summary, General Conclusions and Future Research

The results of the current investigations represent, to the knowledge of the author, the first reported successful *in vitro* propagation of *Monarda*, the first reported case of induced tetraploidy in *Monarda* and the first reported values, for both diploid and tetraploid, of TPC and %AA of *Monarda* leaf extracts. Two species of the genus were chosen for this study, *M. punctata* and *M. fistulosa*, because they are native to Ontario, have attractive and unique flowers known medicinal compounds in their essential oils and are from the two different sub-sections of the genus. These two sub-sections, *Cheilyctis* and *Fistulosa*, are differentiated based on several morphological characteristics, and different chromosome numbers. *M. punctata* has a diploid chromosome number of 22 while *M. fistulosa's* chromosome number is 36 (Scora, 1986a).

Both *M. punctata* and *M. fistulosa* had similar responses to *in vitro* propagation. Of the three explants tested both species had much higher rates of multiplication when nodal sections were used as the explant donor. Nodal sections continued to increase the number of shoots produced as the concentration of BAP continued to increase to 25µM. This is contrary to reports of other species in the same family (Lamiaceae) in which lower optimum concentrations between 1- 9 µM BAP (Kintzios *et al.*, 2004; Fracaro and Echeverrigaray, 2000; Zuzarte *et al.*, 2010) were found.

While the response to *in vitro* culture of both species was generally positive, a large amount of variation was observed as a result of both the age and genotype of the initial explant plant source. Often, of four nodal sections contained on the same petri dish, one would tissue would appear healthy and prolific while the remaining three sections would go brown and die before regenerating a single plantlet. This variation resulted in a lack of statistical significance

between the BAP concentrations tested and may have indirectly altered the interpretation of the data. Both species also demonstrated difficulty rooting *in vitro*, however with the use of rooting powder unrooted plantlets were successfully acclimatized to the greenhouse with 81% survival rate in *M. punctata* and 87% in *M. fistulosa*.

This study exemplified the issues that can arise when beginning tissue culture with a wild heterozygous population. Further work is needed to identify elite germplasm of *M. punctata* and *M. fistulosa* which are better adapted for initiation of *in vitro* cultures. Investigations into the optimal age of a nodal section for an explant donor would also be interesting. Finally, while the reported tissue culture system is both function and efficient, further exploration and optimization of the system is warranted to increase the multiplication rate and root production. Suggested areas for further research included the addition of an auxin source into the medium, a liquid bioreactor and the addition of phenolic and cytokinin inhibitors such as 2-aminoindane-2-phosphonic acid (AIP) and 2,6-diaminopurine.

The induction of autotetraploidy *in vitro* using oryzalin and trifluralin was very successful in both species. A total of 43 *M. punctata* and 124 *M. fistulosa* tetraploid plantlets were successfully identified using flow cytometry and the DNA content of both *Monarda* species was reported. A good correlation between stomatal length and ploidy level was found and could be used as a screening tool in future research. There have been several investigations which have found a correlation between the two characteristics (Cohen and Yao, 1996; Stanys *et al.*, 2006); however, an increase in ploidy level does not always result in an increased stomatal pore size. As with the initial response to *in vitro* propagation, both species showed considerable variation for polyploidization between blocks in the same treatment. This is again possibly related to the initial heterozygous nature of the explant donors.

During the study several diploids and tetraploids with the same parentage were produced and allowed for the study of genome duplication on physiological traits for both species. These paired cytotypes then underwent leaf extractions and were tested for their total phenolic and percentage antioxidant activity using recognized and standard assays. It was in these two assays where the response of *M. punctata* and *M. fistulosa* were greatly different from one another. *M. punctata* demonstrated an increase in TPC with an increase in ploidy level, but %AA was unaffected. In contrast *M. fistulosa's* showed a general decrease of TPC and %AA with increasing ploidy level, although one genotype did show in an increase in TPC and %AA. Reports of TPC and %AA comparisons between genetically paired diploid and tetraploid cytotypes are currently limited and these results are among the few to investigate this potential. Some studies examined the effects of ploidy on commercially available cultivars with varying parentage (Ciccoritti *et al.*, 2012), while a second study was only able to examine four related pairs grown from seed in an outdoor garden (Xie *et al.*, 2012). The study completed in ginger (Sanwal *et al.*, 2010) was the most robust study although its results were not able to provide insight into the underlying cause.

The results of the TPC and %AA assay suggest that the potential for differences in essential oils may exist and warrants further investigations. Firstly the extraction of *M. punctata* and *M. fistulosa* essential oils and identification and quantification of its components would be enlightening. Secondly, inducing tetraploidy in more species of *Monarda* from the various sub-sections and correlating the TPC and %AA results to chromosome number could yield potentially exciting results.

Finally, further investigations into physiological and morphological traits which were not examined in the current study would expand our understanding of induced tetraploidy. How

these two species and their cytotypes would react under different environmental stresses, such as drought or cold temperature is of great interest. As well as classifying morphological differences between body size and growth habit as well as vegetative and reproductive characteristics. If the major oil constituent had been identified it may be possible to relate the change in body size to the essential oil components as reported by Lavania *et al.*, 2012.

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APPENDIX A: ANOVA TABLES

Table A.1. Variance analysis of the effect of varying levels of BAP on the average number of shoots produced from nodal explants of *M. punctata.*

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	0	.	.	.
Residual	0.001640	0.000255	6.44	<.0001
Effect	Num DF	Den DF	*F* value	Pr> *F*
Concentration	6	83	4.97	0.0002
Explant	2	83	14.62	<.0001
Leaf vs Petiole	(1)	(83)	0.02	0.8926
Leaf vs Node	(1)	(83)	22.31	<.0001
Petiole vs Node	(1)	(83)	21.42	<.0001
Explant*Concentration	12	83	0.88	0.5702

Table A.2. Variance analysis of the effect of two different light regimes on the regeneration of *M. punctata* on 25µM BAP for twelve weeks from nodal sections.

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	0	.	.	.
Residual	4.1485	2.0742	2.00	0.0228
Effect	Num DF	Den DF	*F* value	Pr> *F*
Light treatment	1	4	1.19	0.3362

Table A.3. Variance analysis of the effect of two different light regimes on the regeneration of *M. fistulosa* on 25µM BAP for twelve weeks from nodal sections.

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	0.2531	0.6163	0.41	0.3406
Residual	0.9531	0.6740	1.41	0.0786
Effect	Num DF	Den DF	*F* value	Pr> *F*
Light treatment	1	4	18.07	0.0131

Table A.4. Variance analysis of the effect of varying levels of BAP on the average number of shoots produced from nodal explants of *M. punctata.*

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	0.000071	0.000164	0.43	0.3321
Residual	0.001051	0.000309	3.40	0.0003
Effect	Num DF	Den DF	*F* value	Pr> *F*
Concentration	6	23	2.18	0.0830
0 vs 1	(1)	(23)	0.04	0.8352
0 vs 5	(1)	(23)	1.38	0.2522
0 vs 10	(1)	(23)	1.97	0.1741
0 vs 15	(1)	(23)	2.20	0.1514
0 vs 20	(1)	(23)	0.17	0.6865
0 vs 25	(1)	(23)	3.19	0.0874

Table A.5. Variance analysis of the effect of varying levels of BAP on the percentage of nodal explants producing shoots in *M. punctata.*

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	0	.	.	.
Residual	0.05949	0.01590	3.74	<.0.001
Effect	Num DF	Den DF	*F* value	Pr> *F*
Concentration	6	28	1.55	0.1984
0 vs 1	(1)	(28)	0.17	0.6799
0 vs 5	(1)	(28)	5.69	0.0240
0 vs 10	(1)	(28)	0.19	0.6703
0 vs 15	(1)	(28)	0.31	0.5841
0 vs 20	(1)	(28)	0.46	0.5035
0 vs 25	(1)	(28)	0.37	0.5479

Table A.6. Variance analysis of the effect of varying levels of BAP on the average number of shoots produced from nodal explants of *M. punctata* which were able to regenerate over 12 weeks.

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	0.000544	0.000688	0.79	0.2146
Residual	0.0029	0.000837	3.46	0.0003
Effect	Num DF	Den DF	*F* value	Pr> *F*
Concentration	6	24	1.28	0.3020
0 vs 1	(1)	(24)	0.25	0.6219
0 vs 5	(1)	(24)	0.19	0.6674
0 vs 10	(1)	(24)	1.00	0.3265
0 vs 15	(1)	(24)	0.63	0.4356
0 vs 20	(1)	(24)	0.27	0.6054
0 vs 25	(1)	(24)	1.53	0.2278

Table A.7. Variance analysis of the effect of varying levels of BAP on the total number of shoots produced by 12 nodal explants of *M. punctata* over a 12 week period.

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	6.5868	10.7215	0.61	0.2695
Residual	56.9512	16.7472	3.40	0.0003
Effect	Num DF	Den DF	*F* value	Pr> *F*
Concentration	6	23	2.04	0.1004
0 vs 1	(1)	(23)	0.01	0.9235
0 vs 5	(1)	(23)	1.31	0.2641
0 vs 10	(1)	(23)	1.08	0.3084
0 vs 15	(1)	(23)	3.07	0.0930
0 vs 20	(1)	(23)	0.12	0.7297
0 vs 25	(1)	(23)	3.39	0.0787

Table A.8. Variance analysis of the effect of varying levels of BAP on the average number of shoots produced from nodal explants of *M. fistulosa.*

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	0	.	.	.
Residual	0.002686	0.000829	3.24	0.0006
Effect	Num DF	Den DF	*F* value	Pr> *F*
Concentration	6	21	1.06	0.4147
0 vs 1	(1)	(21)	0.23	0.633
0 vs 5	(1)	(21)	0.00	0.9893
0 vs 10	(1)	(21)	0.27	0.6120
0 vs 15	(1)	(21)	0.03	0.8620
0 vs 20	(1)	(21)	2.67	0.1174
0 vs 25	(1)	(21)	2.93	0.1017

Table A.9. Variance analysis of the effect of varying levels of BAP on the percentage of nodal explants producing shoots in *M. fistulosa.*

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	0.01791	0.02223	0.81	0.2102
Residual	0.06341	0.02114	3.00	0.0013
Effect	Num DF	Den DF	*F* value	Pr> *F*
Concentration	6	18	1.23	0.3380
0 vs 1	(1)	(18)	0.08	0.7766
0 vs 5	(1)	(18)	0.00	0.9813
0 vs 10	(1)	(18)	0.83	0.3747
0 vs 15	(1)	(18)	0.23	0.6406
0 vs 20	(1)	(18)	3.47	0.0790
0 vs 25	(1)	(18)	0.76	0.3937

Table A.10. Variance analysis of the effect of varying levels of BAP on the average number of shoots produced from nodal explants of *M. fistulosa* which were able to regenerate over 12 weeks.

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	0	.	.	.
Residual	0.002285	0.000705	3.24	0.0006
Effect	Num DF	Den DF	*F* value	Pr> *F*
Concentration	6	21	0.74	0.6230
0 vs 1	(1)	(21)	0.49	0.4905
0 vs 5	(1)	(21)	0.00	0.9652
0 vs 10	(1)	(21)	0.06	0.8034
0 vs 15	(1)	(21)	0.51	0.4839
0 vs 20	(1)	(21)	1.53	0.2299
0 vs 25	(1)	(21)	2.60	0.1221

Table A.11. Variance analysis of the effect of varying levels of BAP on the total number of shoots produced by 12 nodal explants of *M. fistulosa* over a 12 week period.

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	2.1299	15.0749	0.14	0.4438
Residual	84.6001	31.2091	2.71	0.0034
Effect	Num DF	Den DF	*F* value	Pr> *F*
Concentration	6	15	0.45	0.8320
0 vs 1	(1)	(15)	0.85	0.3698
0 vs 5	(1)	(15)	0.01	0.9146
0 vs 10	(1)	(15)	0.00	0.9479
0 vs 15	(1)	(15)	0.04	0.8375
0 vs 20	(1)	(15)	0.73	0.4068
0 vs 25	(1)	(15)	1.00	0.3343

Table A.12. Variance analysis of the positional effect of *M. punctata's* nodal section on the regeneration capacity of explants *in vitro.*

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	1.6705	0.4743	1.71	0.0432
Residual	2.3561	0.5800	4.06	<.0001
Effect	Num DF	Den DF	*F* value	Pr> *F*
Position	3	33	1.52	0.2273

Table A.13. Variance analysis of the genotype effect of *M. punctata's* nodal section on the regeneration capacity of explants *in vitro.*

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	0.1023	0.2486	0.41	0.3404
Residual	2.3561	3.800	4.06	<.0001
Effect	Num DF	Den DF	*F* value	Pr> *F*
Genotype	11	33	3.84	0.0013

Table A.14. Variance analysis of the positional effect of *M.fistulosa's* nodal section on the regeneration capacity of explants *in vitro.*

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	5.1932	2.8389	1.83	0.033
Residual	5.6572	1.3927	4.06	<.0001
Effect	Num DF	Den DF	*F* value	Pr> *F*
Position	3	33	1.83	0.1609

Table A.15. Variance analysis of the genotype effect of *M.fistulosa's* nodal section on the regeneration capacity of explants *in vitro.*

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	0.3917	0.7140	0.55	0.2918
Residual	5.6572	1.3927	1.06	<.0001
Effect	Num DF	Den DF	*F* value	Pr> *F*
Genotype	11	33	4.67	0.0003

Table A.16. Variance analysis of the effect of rooting media on the total number of roots produced by *M. punctata* plantlets in six weeks.

Source	*Df*	Type III SS	MS	F Value	P>F
Treatment	13	201.9495	15.5346	2.41	0.0067
AC vs no AC	(1)	29.8897	29.8897	4.64	0.0334
1/2MS vs MS	(1)	4.9730	4.9730	0.77	0.3814
Error	110	708.2683	6.4388		

Table A.17. Variance analysis of the effect of rooting media on the length of the longest root produced by *M. punctata* plantlets after six weeks.

Source	*Df*	Type III SS	MS	F Value	P>F
Treatment	13	51.4204	3.9554	1.64	0.0813
AC vs no AC	(1)	11.2150	11.2150	4.66	0.0327
1/2MS vs MS	(1)	0.03875	0.03875	0.02	0.8992
Error	128	301.9506	2.4058		

Table A.18. Variance analysis of the effect of rooting media on the change in *M. punctata* plantlets height after six weeks.

Source	*Df*	Type III SS	MS	F Value	P>F
Treatment	13	219.5535	16.8887	2.48	0.0048
AC vs no AC	(1)	0.354404	0.354404	0.05	0.8199
1/2MS vs MS	(1)	30.8286	30.8286	4.53	0.0353
Error	128	871.5658			

Table A.19. Variance analysis of the effect of rooting media on the change of the number of nodes in *M. punctata* plantlets after six weeks.

Source	*Df*	Type III SS	MS	F Value	P>F
Treatment	13	68.7732	5.2902	1.60	0.0919
AC vs no AC	(1)	1.4365	1.4365	0.44	0.5104
1/2MS vs MS	(1)	25.2158	25.2158	7.65	0.0065
Error	128	422.1000			

Table A.20. Variance analysis of the effect of rooting media on the total number of roots produced by *M. fistulosa* plantlets in six weeks.

Source	*Df*	Type III SS	MS	F Value	P>F
Treatment	13	1289.9268	99.2251	12.40	<.0001
AC vs no AC	(1)	174.1512	174.1512	21.76	<.0001
1/2MS vs MS	(1)	13.1722	13.1722	1.65	0.2009
Error	203	1624.2944	8.0015		

Table A.21. Variance analysis of the effect of rooting media on the length of the longest root produced by *M. fistulosa* plantlets after six weeks.

Source	*Df*	Type III SS	MS	F Value	P>F
Treatment	13	164.1776	12.6290	4.30	<.0001
AC vs no AC	(1)	0.002171	0.002171	0.00	0.9783
1/2MS vs MS	(1)	1.5185	1.5185	0.52	0.4726
Error	227	666.0755	2.9342		

Table A.22. Variance analysis of the effect of rooting media on the change in *M. fistulosa* plantlets height after six weeks.

Source	*Df*	Type III SS	MS	F Value	P>F
Treatment	13	2231.5433	171.6571	14.50	<.0001
AC vs no AC	(1)	371.9681	371.9681	31.43	<.0001
1/2MS vs MS	(1)	401.9442	401.9442	33.96	<.0001
Error	227	2686.4145	11.8344		

Table A.23. Variance analysis of the effect of rooting media on the change of the number of nodes in *M. fistulosa* plantlets after six weeks.

Source	*Df*	Type III SS	MS	F Value	P>F
Treatment	13	196.8861	15.1451	7.05	<.0001
AC vs no AC	(1)	36.0657	36.0657	16.80	<.0001
1/2MS vs MS	(1)	34.9480	34.9480	16.28	<.0001
Error	227	487.4126	2.1472		

Table A.24. Variance analysis of total number of *M. punctata in vitro* plantlets produced when exposed to varying concentrations and chemicals over one, three or six days.

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	0.00896	0.02574	0.35	0.3638
Residual	1.2504	0.1325	9.44	<.0001
Effect	Num DF	Den DF	*F* value	Pr> *F*
Chemical	1	178	4.93	0.0277
Concentration	7	178	2.68	0.0117
Duration	2	178	8.87	0.0002
Chem*Conc	7	178	1.07	0.3856
Chem*Dur	2	178	1.16	0.3167
Dur*Conc	14	178	1.43	0.1444
Chem*Dur*Conc	14	178	0.46	0.9532

Table A.25. Variance analysis of total number of *M. fistulosa in vitro* plantlets produced when exposed to varying concentrations and chemicals over one, three or six days.

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	0.009621	0.02402	0.4	0.3444
Residual	1.0949	0.1183	9.25	<.0001
Effect	Num DF	Den DF	*F* value	Pr> *F*
Chemical	1	171	0.49	0.4861
Concentration	7	171	1.88	0.0762
Duration	2	171	26.37	<.0001
Chem*Conc	7	171	0.50	0.8313
Chem*Dur	2	171	0.39	0.6756
Dur*Conc	14	171	0.69	0.7824
Chem*Dur*Conc	14	171	1.44	0.1406

Table A.26. Variance analysis of the interaction of ploidy (tetraploid, diploid, chimeric) and genotype from 10 *M. punctata* genotypes on the GAE (mg/mL) of leaf extracts as calculated using the Folin-Ciocalteau assay.

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	0	.	.	.
Residual	0.8738	0.1076	8.12	<.0001
Effect	Num DF	Den DF	*F* value	Pr> *F*
Genotype*Ploidy	23	131	9.94	<.0001
Genotype 1	(1)	(131)	1.29	0.2579
Genotype 2	(1)	(131)	10.85	0.0013
Genotype 3	(1)	(131)	0.97	0.3275
Genotype 4	(1)	(131)	1.29	0.2581
Genotype 5	(1)	(131)	2.25	0.1360
Genotype 6	(1)	(131)	4.65	0.0329
Genotype 7	(1)	(131)	0.03	0.8741
Genotype 8	(1)	(131)	9.49	0.0025
Genotype 9	(1)	(131)	35.49	<.0001
Genotype 10	(1)	(131)	2.28	0.1332

Table A.27. Variance analysis of the individual effects of ploidy (tetraploid, diploid, chimeric) and genotype from 10 *M. punctata* genotypes on the GAE (mg/mL) of leaf extracts as calculated using the Folin-Ciocalteau assay. .

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	0	.	.	.
Residual	1.1416	0.1345	8.49	<.0001
Effect	Num DF	Den DF	*F* value	Pr> *F*
Ploidy	2	143	18.66	<.0001
Genotype	9	143	12.3	<.0001

Table A.28. Variance analysis of the interaction of ploidy (tetraploid, diploid, chimeric) and genotype from 10 *M. fistulosa* genotypes on the GAE (mg/mL) of leaf extracts as calculated using the Folin-Ciocalteau assay.

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	0.05957	0.1213	0.49	0.3116
Residual	1.5682	0.2207	7.11	<.0001
Effect	Num DF	Den DF	*F* value	Pr> *F*
Genotype*Ploidy	17	101	9.63	<.0001
Genotype 1	(1)	(101)	4.69	0.0327
Genotype 2	(1)	(101)	6.24	0.0141
Genotype 3	(1)	(101)	0.34	0.5626
Genotype 4	(1)	(101)	1.39	0.2416
Genotype 5	(1)	(101)	2.7	0.1032
Genotype 6	(1)	(101)	7.68	0.0067
Genotype 7	(1)	(101)	25.73	<.0001
Genotype 8	(1)	(101)	10.12	0.0019
Genotype 9	(1)	(101)	14.77	0.0002

Table A.29. Variance analysis of the individual effects of ploidy (tetraploid, diploid, chimeric) and genotype from 10 *M. fistulosa* genotypes on the GAE (mg/mL) of leaf extracts as calculated using the Folin-Ciocalteau assay. .

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	0.05122	0.1213	0.42	0.3364
Residual	2.0691	0.2816	7.35	<.0001
Effect	Num DF	Den DF	*F* value	Pr> *F*
Ploidy	2	108	12.18	<.0001
Genotype	8	108	11.01	<.0001

Table A.30. Variance analysis of the interaction of ploidy (tetraploid, diploid, chimeric) and genotype from 10 *M. punctata* genotypes on the % AA of leaf extracts as calculated using the DPPH assay.

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	32.8502	47.0275	0.7	0.2424
Residual	31.4528	3.8863	8.09	<.0001
Effect	Num DF	Den DF	*F* value	Pr> *F*
Genotype*Ploidy	23	131	4.07	<.0001
Genotype 1	(1)	(131)	0.02	0.8902
Genotype 2	(1)	(131)	0.19	0.6608
Genotype 3	(1)	(131)	0.47	0.4959
Genotype 4	(1)	(131)	2.21	0.1397
Genotype 5	(1)	(131)	0.26	0.6082
Genotype 6	(1)	(131)	0.27	0.6027
Genotype 7	(1)	(131)	1.48	0.2259
Genotype 8	(1)	(131)	0.06	0.8128
Genotype 9	(1)	(131)	0.6	0.4396
Genotype 10	(1)	(131)	0.01	0.9099

Table A.31. Variance analysis of the individual effects of ploidy (tetraploid, diploid, chimeric) and genotype from 10 *M. punctata* genotypes on the % AA of leaf extracts as calculated using the DPPH assay.

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	32.8346	47.0275	0.7	0.2425
Residual	32.6701	3.836	8.46	<.0001
Effect	Num DF	Den DF	*F* value	Pr> *F*
Ploidy	2	143	0.97	0.3825
Genotype	9	143	7.23	<.0001

Table A.32. Variance analysis of the interaction of ploidy (tetraploid, diploid, chimeric) and genotype from 10 *M. fistulosa* genotypes on the % AA of leaf extracts as calculated using the DPPH assay.

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	10.3759	14.8131	0.7	0.2418
Residual	5.9102	0.8317	7.11	<.0001
Effect	Num DF	Den DF	*F* value	Pr> *F*
Genotype*Ploidy	17	101	11.4	<.0001
Genotype 1	(1)	(101)	28.24	<.0001
Genotype 2	(1)	(101)	5.37	0.0225
Genotype 3	(1)	(101)	19.09	<.0001
Genotype 4	(1)	(101)	2.25	0.1367
Genotype 5	(1)	(101)	0.54	0.4628
Genotype 6	(1)	(101)	7.16	0.0087
Genotype 7	(1)	(101)	1.76	0.1878
Genotype 8	(1)	(101)	22.96	<.0001
Genotype 9	(1)	(101)	9.67	0.0024

Table A.33. Variance analysis of the individual effects of ploidy (tetraploid, diploid, chimeric) and genotype from 10 *M. fistulosa* genotypes on the % AA of leaf extracts as calculated using the DPPH assay.

Cov Parm	Estimate	Standard Error	*Z* value	Pr *Z*
Block	10.319	14.8131	0.7	0.26430
Residual	9.3287	1.2695	7.35	<.0001
Effect	Num DF	Den DF	*F* value	Pr> *F*
Ploidy	2	108	8.74	0.0003
Genotype	8	108	7.14	<.0001

APPENDIX B: SUPPLEMENTARY TABLES

Table B.1. Excised data demonstrating the extremes of the variability observed between individual nodal explants of *M. punctata* on the same petri plate across all seven BAP concentrations tested.

Concentration	Block	Plate	Explant	Total Shoots
0	5	1	1	6
			2	11
			3	0
			4	0
1	2	1	1	1
			2	2
			3	3
			4	9
5	5	1	1	1
			2	2
			3	0
			4	0
10	5	2	1	4
			2	11
			3	0
			4	0
15	4	1	1	13
			2	2
			3	0
			4	0
20	3	1	1	11
			2	0
			3	0
			4	0
25	3	1	1	14
			2	2
			3	0
			4	0

Table B.2. Excised data demonstrating the extremes of the variability observed between individual nodal explants of *M. fistulosa* on the same petri plate across all seven BAP concentrations tested.

Concentration	Block	Plate	Explant	Total Shoots
0	2	1	1	3
			2	4
			3	1
			4	0
1	1	2	1	2
			2	2
			3	15
			4	2
5	2	2	1	11
			2	0
			3	0
			4	0
10	1	1	1	3
			2	4
			3	10
			4	1
15	5	1	1	11
			2	2
			3	4
			4	0
20	5	1	1	1
			2	1
			3	18
			4	14
25	1	2	1	2
			2	2
			3	2
			4	17

Table B.3. Volume of Oryzalin or Trifluralin 30 mM stock solutions added to basal MS medium in order to achieve different concentrations of induction media.

Final Concentration(µM)	Trifluralin/Oryzalin stock (mL)	DMSO (mL)	MS (mL)
0		4.8[z]	395.2
1	0.04	4.76	395.2
5	0.2	4.6	395.2
15	0.6	4.2	395.2
30	1.2	3.6	395.2
60	2.4	2.4	395.2
90	3.6	1.2	395.2
120	4.8		395.2

[z] DMSO was added only to Oryzalin control plates. Trifluralin control plates contained 400 mL of MS media with no addition of DMSO or chemicals.

Table B.4. List of plant genotypes used in the current investigation and their corresponding ID number and ploidy level.

M. punctata			*M. fistulosa*		
ID	Genotype	Ploidy	ID	Genotype	Ploidy
151	1	D[z]	340	1	D
47	1	T	295A	1	T
247	1	C	381	2	T
153	2	D	332C	2	D
18	2	C	352	3	D
5	2	T	303	3	T
66	3	T	313A	4	D
209B	3	C	225A	4	T
242B	3	D	331A	5	D
187	4	T	320C	5	T
271	4	T	314B	6	D
210A	4	D	322A	6	T
262	5	D	354	7	C
245A	5	T	221C	7	T
147	5	C	302	7	T
65	6	T	399	8	T
206B	6	D	37	8	D
266	7	D	290B	9	T
274	7	T	318C	9	T
6	8	T	329C	9	D
157	8	D	39	9	D
3	9	T			
25	9	T			
17	9	D			
281	10	C			
233	10	T			

[z] D= diploid; T= tetraploid; C= chimeric.