CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Tropical AgriSciences

Department of Crop Sciences and Agroforestry

In vitro induction of polyploidy in *Thymus vulgaris L.*

Bachelor Thesis

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Declaration

I hereby declare that this thesis entitled; *In vitro* induction of polyploidy in *Thymus vulgaris L*. is my own work and all the sources have been quoted and acknowledged by means of complete references.

I further declare that the work was submitted for the first time at the Czech University of Life Sciences Prague / Faculty Tropical Agrisciences towards the Bachelor degree of Crop Sciences and Agroforestry and that it has never been submitted to any other university/faculty for the purpose of obtaining a degree.

In Prague: / / 2015 **Yamen Homaidan Shmeit**

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Abstract

Thymus vulgaris L. is a species of subshrubs in the family *Lamiaceae* with medicinal and pharmaceutical utilization. As a medicinal plant it is used in the treatment of headaches, diarrhea, constipation, warts, and kidney malfunction. It also contains thymohydroquinone (THQ), dithymoquinone (DTQ) and a slightly high amount of thymoquinone (TQ), a compound with anticancer, antimicrobial and antioxidant properties. As well as hepatoproptective and immunomodulatory effects.

The aim of the study was to obtain tetraploid plants (2n=60) chromosome number from diploid plants (2n=30) using *in vitro* induced polyploidization on species of *Thymus vulgaris L.*

Nodal cultures were used for polyploidization. Nodal segments were exposed to a solution of oryzalin (antimitotic agent) in concentrations of 20, 40, 60 and 80 μM with treatment duration of 24 h and 48 h. After nodal segments were cultivated on MS medium free of plant growth regulators and passaged three times along duration of two months. Ploidy level of affected plants was determined by flow cytometry.

Five tertaploid plants (2n=60) were obtained. Percent of success was 4.7 %. Plants were obtained in concentrations of 60 μM (1 plant) and 80 μM (4 plants). As for morphological changes tetraploid plants had significantly thicker stems and a darker green color especially in the leaves. There was also an increase in leaf thickness and number per plant compared to the control plants. Obtained results also confirmed that oryzalin could be used as an antimitotic agent for obtaining polyploidy plants in *Thymus vulgaris L.*

Polyploidization could have a positive effect on both morphological and biochemical properties.

Key words: Dithimohydroquinone – *in vitro* – *Lamiaceae* – nodal segments – oryzalin – polyploidization – *Thymus vulgaris L.* – Thymohydroquinone – Thymoquinone.

Abstrakt

Thymus vulgaris L. je polokeř z čeledi *Lamiaceae* s léčebným a farmaceutickým využitím. Jako léčivá rostlina je využíván při léčbě bolesti hlavy, průjmu, zácpy, bradavic a při selhání ledvin. Obsahuje thymohydroquinone (THQ), dithymoquinone (DTQ) a malé množství thymoquinone (TQ), sloučeniny s protinádorovými, antimikrobiálními a antioxidačními vlastnostmi. Stejně tak má i hepatoprotektivní a imunomodulační účinky.

Cílem této studie bylo získat tetraploidní rostliny (2n=60) z diploidních rostlin (2n=30) pomocí *in vitro* indukované polyploidie u *Thymus vulgaris L.*

Pro indukci polyploidie byly použity nodální segmenty, které byly vystaveny působení oryzalinu (antimitotického činidla) v koncentracích 20, 40, 60 a 80 μM po dobu 24 a 48 h. Poté byly nodální segmenty kultivovány na MS mediu bez růstových regulátorů a množeny třikrát během dvou měsíců. Úroveň ploidie byla stanovena pomocí flow cytometrie.

Bylo získáno 5 tetraploidních rostlin (2n=60). Celkové procento úspěšnosti polyploidizace bylo 4,7%. Polyploidní rostliny byly získány v koncentraci oryzalinu 60 μM (1 rostlina) a 80 μM (4 rostliny). U tetraploidních rostlin bylo dosaženo morfologických změn. Rostliny měly významně silnější stonky a tmavší zelenou barvu, zejména v listech. Došlo také k nárůstu tloušťky listů i v počtu listů na rostlinu ve srovnání s kontrolními rostlinami. Získané výsledky potvrzují, že oryzalin je efektivním antimitotickým činidlem pro získání polyploidních rostlin u *Thymus vulgaris L.*

Polyploidizace může mít pozitivní efekt na morphologické I biochemické vlastnosti rostlin.

Klíčová slova: Dithimohydroquinone – *in vitro* – *Lamiaceae* – nodální segment – oryzalin – polyploidizace – *Thymus vulgaris L.* – Thymohydroquinone – Thymoquinone.

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LIST OF ABBREVIATION

FCM	**Flow cytometer**
IC50	**Half maximal inhibitory concentraion**
KOH	**Potassium hydroxide**
MS	**Murashige and Akoog (1962) medium**
MTT	**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide**
PBMCs	**Peripheral Blood Mononuclear Cells**

1. Introduction

The genus *Thymus* comprises important species, including *Thymus vulgaris L.,* an angiosperm in the *Lamiaceae* family. The genus comprises of around 350 species of perennial, aromatic herbs and subshrubs and is predominantly native to the Mediterranean region, Asia, Southern Europe and North Africa (Maksimovic et al., 2008). Thyme is a semi-woody sub shrub with aromatic, oblong, oval, dark-green leaves that vary in length from 6 to 12 mm. The plant also produces pink to violet or pink to white clusters of flowers. It is an aromatic and medicinal plant, used for medicinal and spice purposes almost everywhere in the world. The herb has a pungent taste. Essential oils extracted from fresh leaves and flowers can be used as aroma additives in food, pharmaceuticals and cosmetics (Senatore, 1996; Simon et al., 1999; Mendes and Romano, 2004).

Thymus species are considered as medicinal plants due to their pharmacological and biological properties. In native medicine, flowering parts and leaves of *Thymus* species have been extensively used as herbal tea. The carminative herb is used to fight flatulence, as an antitussive for alleviating or suppressing coughing, and as an antiseptic and for treating colds (Karaman et al., 2001; Rasooli and Mirmostafa, 2003; Rota et al., 2004; Sotomayor et al., 2004; Maksimovic et al., 2008; Rota et al., 2008). It is also used as a medicinal plant in the treatment of headaches, diarrhea, constipation, warts, and kidney malfunction (Simon et al., 1999). According to the literature reports the composition and biological activities of essential oil from various *Thymus* species are rarely investigated.

Reading Curtis et al. (1996) showed these compounds to have bactericidal and fungicidal properties. It is being used in both Homeopathic and Allopathic medicine. In early pharmacological works, (Economou et al., 1991; Deighton et al., 1993; Schwarz et al., 1996) reported the extracts of thyme to show antioxidant activity; estrogen and progestin bioactivity (Mendes and Romano, 2004), and antimutagenic activity (Miura and Nakatani, 1989). Also three biphenyl compounds in thyme were identified as deodorant compounds (Nakatani et al., 1989).

Táborský et al., (2012) proved *Thymus vulgaris L*. to contain thymohydroquinone (THQ), dithymoquinone (DTQ) and a slightly high amount of Thymoquinone (TQ), a

compound with anticancer, antimicrobial, and antioxidant properties. As well as hepatoprotective and immunomodulatory effects.

In vitro technologies like micropropagation of *Thymus vulgaris L.* exist but fewer studies about *in vitro* polyploidization are found. Such studies could lead to higher plant yields thus leading to an increase in production and could lead to higher content of essential oils per plant. Thyme is an important European culinary herb and has a high economic importance in countries that produce it such as Italy, Spain, France, Greece, Switzerland, Portugal, Thailand, Singapore and India (FAO and WHO, 2013). Due to the increasing use of thyme as a medicinal plant and because of recent studies about the plant's potential in food industry as an antioxidant, we are interested in using *in vitro* technologies like polyploidization.

In this study, main objective of polyploidization is to achieve plants with higher ploidy level for plant character changes which can significantly influence the habit and utilization of these plants. Polyploidization could have a positive effect on both morphological and biochemical properties.

2. Literature Review

Thymus vulgaris L. (thyme), locally known as "zaatar", a member of the *Lamiaceae* family, is a pleasant aromatic herb and sub shrub, which grows in several regions in the world (Davis, 1982). Maksimovic et al. (2008) mentions that the genus *Thymus* is predominantly found in the Mediterranean region, Southern Europe and North Africa; It is commonly known that the composition of the essential oils determines the specific aroma of plants and flavor of condiments (Martins et al., 1999).

Thyme leaves are small in size, grey to green in color and come in opposite, linear oblong to lanceolate form with recurved margines. The inflorescence is capitate and flowers are violet to white, two-lipped, around 5 mm long with a hairy, glandular, leaf like calyx and bear quadruplet nutlet fruits (Fig.1).

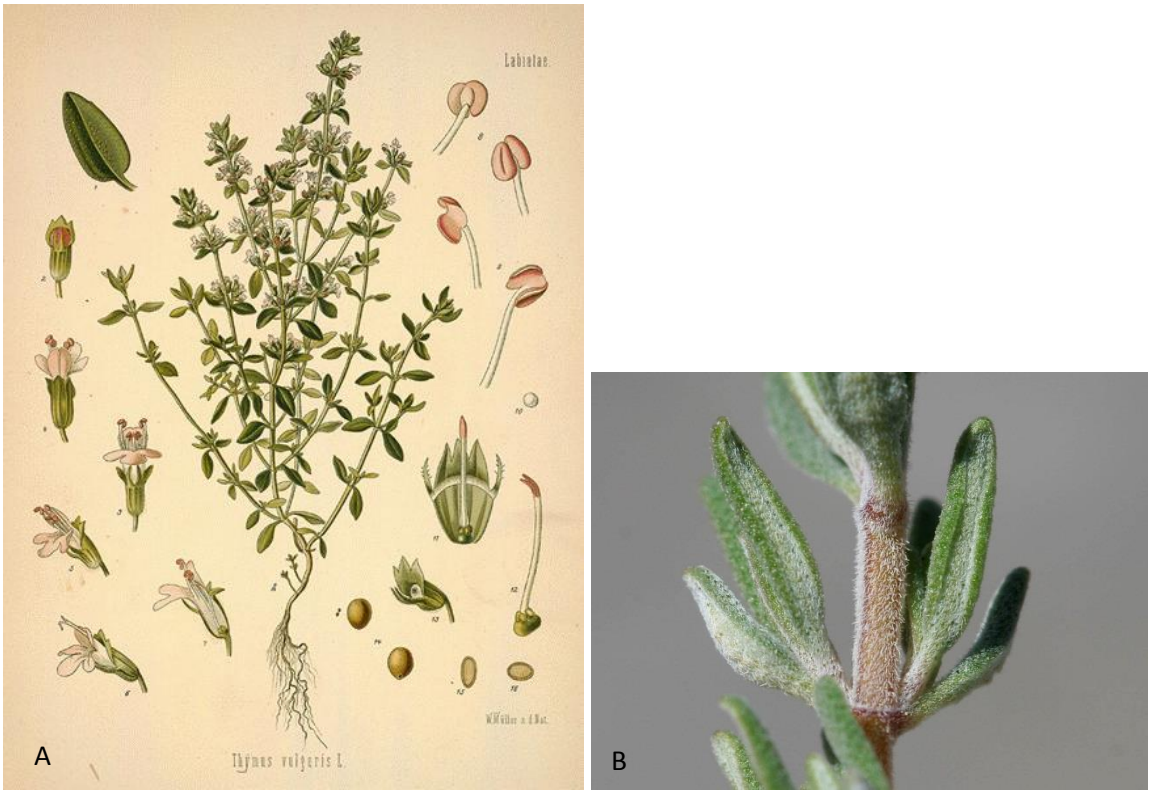

Figure 1: Morphological details of Thyme

Source: A - Grieve (2014); B – Bausselen (2015)

2.1 Botanic Classification of *Thymus vulgaris L.*

Thymus vulgaris L. constitutes of a stable karyological systematic unit in which botanists can easily taxonomically characterize. It is spread in a localized way in Europe and the Mediterranean zone and its existence does not exceed directly boarding regions to the Mediterranean. Several karyological studies held the same results that *Thymus vulgaris L.* has a number of chromosomes of 2n = 30 (Jalas, 1948; Stahl-Biskup and Saez, 2002)

***Thymus vulgaris* can be classified as shown below.**

- Domain: *Eukarya*
- Kingdom: *Plantae*
- Class: *Magnoliopsida*
- Order: *Lamiales*
- Family: *Lamiaceae*
- Genus: *Thymus L.*
- Species: *Thymus vulgaris L.*

2.2 Habitat of *Thymus vulgaris L*

Thymus vulgaris originates from the Mediterranean and grows in North America, Europe and North Africa (Letchamo and Gosselin, 1996). In present time, thyme is cultivated in Iran in large amounts. Thyme proved itself to acquire an important place in expanding world market.

2.3 Reproduction of *Thymus vulgaris L.*

Agricultural factors affect quantity and quality in thyme; spacing and harvesting schedule are very effective factors in this area. Shalby and Razin (1992) cultivated thyme in rows 60 cm apart with inter-row spacing of 15, 30 and 45 cm. In their discussion they stated that the wider the spacing, the greater were the yields of essential oil per plant. Thus close spacing increased the yield of herbage and oil per unit area, but in close spacing the plant could be subject to fungal infections where ideal microclimates for fungi and bacterial infections could form.

Harvesting time is an important factor that influences oil yield. From June to August, plants start to develop pink to white and violet flowers; (McGimpsey et al., 1994) experiments proved most oils produced from flowering plants had oil content at its highest levels at that time. Also similar experiments proved seasonal variation to have significant effects on the yield and composition of thyme oil.

A report on thyme grown in northern Italy indicated that phenol content at full flowering varied from year to year (Piccaglia and Maroti, 1991).

Rey (1991) has shown at the location Arbaz, that the best harvesting time was late September with cutting at 10 cm or 15 cm height. On the other hand, at Bruson cutting in mid-August to a height of 15 cm was the best. Thus harvesting time is strongly influenced by the area of cultivation and its environmental conditions.

2.4 Chemical Composition

Parts of thyme, especially stems and leaves contain volatile oils around 25% with a significant odder and spicy taste. They also contain several phenolic acids and terpenes, the composition and compound structure of essential oils found in thyme is given in Fig. 2.

The main chemical composition in thyme is in its essential oils, which include thymol, carvacrol, borneol, linalool, myrcene, p-cymene and other flavonoids. Other oils in the plant are tannin, saponins and triterpenic acids. Furthermore *Thymus vulgaris L* shows a polymorphic variation in monoterpene production, the presence of intraspecific chemotype variation being common in the genus *Thymus*. Each of the six chemotypes, geraniol (G), α-terpineol (A), thuyanol-4 (U), linalool (L), carvacrol (C), and thymol (T), is named after its dominant monoterpene (Shabnum and Wagay, 2011).

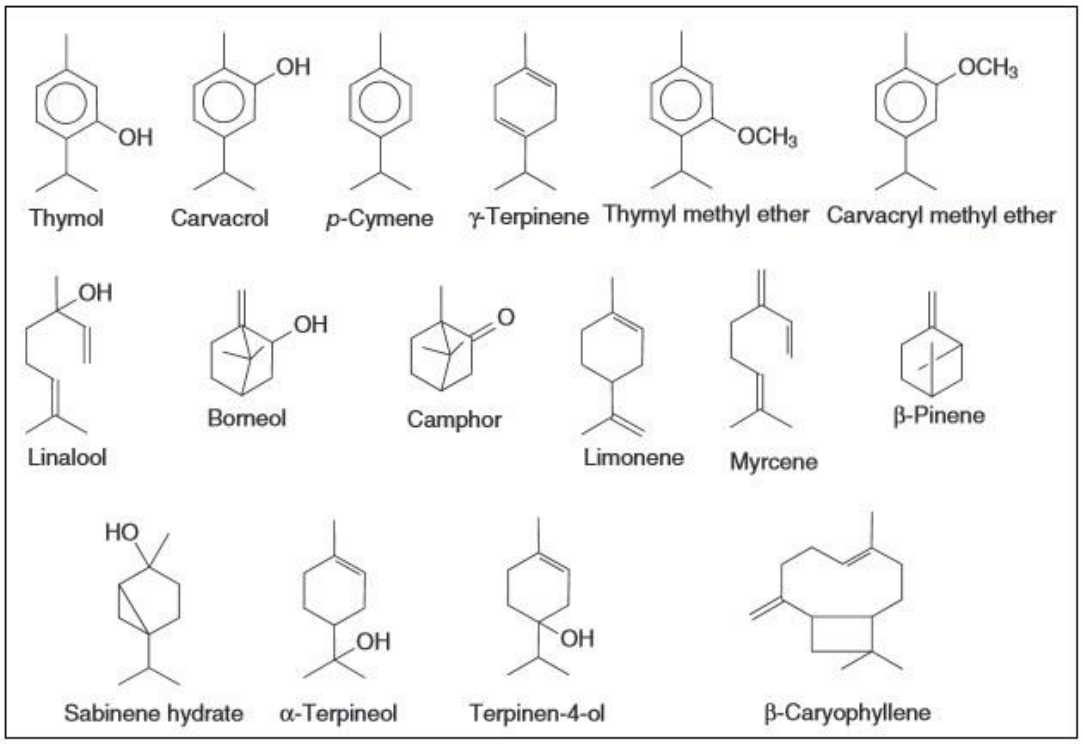

Figure 2: Terpenes in the essential oil of thyme

Source: Stahl-Biskup and venskutonis (2012)

2.5 Medicinal Uses

Recent studies have shown that *Thymus* species have strong antibacterial, antifungal, antiviral, antiparasitic, spasmolytic and antioxidant activities (Stahl-Biskup and Saez, 2002). (Manou et al., 1998) did experiments on thyme oil medicinal and cosmetic purposes: it increases skin natural resistance and adds fragrance to cosmetics. Also (Woodruff, 1995) mentioned that thyme antioxidant is used in food industry where it removes the health risks or side effects of artificial antioxidants. Extracts of thyme are used to treat bronchial infections, reducing flu side effects, throat infections and for asthma chronic inflammatory diseases. Other uses are for allergies and as anthelmintics for expelling parasitic worms. *Thyme* is useful for ringworm, athlete's foot, thrush, and other fungal infections, as well as scabies and lice (Barnes et al., 2002).

These extracts of *Thymus vulgaris L.* are useful in traditional medicine because of their antiasthmatic, bronchodilator, antiseptic, antispasmodic, antitussive, antibacterial,

antifungal and antiviral activities (Marino et al., 1999). These extracts have also shown immunomodulating properties (Ocaña and Reglero, 2012).

Thymus vulgaris L is quoted by various authors for its polyphenol and flavonoid contents such as its potential antioxidant and free radical scavenging, anti-inflammatory, vasorelaxant, anti-platelet, antithrombin, anti-hyperlipidemic and anti-diabetic properties (Miura et al., 2002). Applied to the skin, thyme relieves bites and stings, and relieves sciatica and rheumatic aches and pains.

A recent study (Kensara et al., 2013) also shows that supplementation with *Thymus vulgaris L* as a herbal remedy has shown remarkable antihypertensive effect and marked improvement on hypertension-related biochemical changes and aortic vascular damage in rats.

2.5.1 Anticancer Studies

Some recent studies concerning the cytotoxic effect of *Thymus vulgaris* are mentioned here. In Moroco, Jaafari et al., (2007) published a paper where *Thymus vulgaris L* essential oils as well as two pure products (carvacrol and thymol) were tested for their antitumor activity against P815 mastocytoma cell line using colorimetric MTT assay. While all these products showed a dose dependent cytotoxic effect, the carvacrol (with IC50 less than 0.004 % (v/v)) was the most cytotoxic one compared to the others. The IC50 of thymol was 0.015 % (v/v). Interestingly, when these products were tested against the normal human peripheral blood mononuclear cells (PBMC), they (except thymol) showed a proliferative effect instead of a cytotoxic one. Thymol had cytotoxic effect on the PBMC. The half maximal inhibitory concentration (IC50) is a measure of the inhibiting effectiveness of a substance.

Zu et al. (2010) in China tested the essential oil of *Thymus vulgaris L* for its *in vitro* toxicology against three human cancer cell lines, PC-3, A-549 and MCF-7 cancer cells. *Thymus vulgaris L* essential oil exhibited cytotoxicity towards three human cancer cells. Its IC50 values on PC-3, A549 and MCF-7 tumor cell lines were 0.010 % (v/v), 0.011 % (v/v) and 0.030 % (v/v), respectively.

Another study in Germany was performed by Sertel et al. (2011), where cytotoxicity of *thyme* essential oil was investigated on the head and neck squamous cell carcinoma (HNSCC) cell line. They found that the IC50 of thyme essential oil extract was 369 μg/ mL. Berrington

and Lall (2012) in South Africa published a paper where acetone extract of *T. vulgaris* L. was evaluated for its *in vitro* cytotoxicity against a noncancerous African green monkey kidney (Vero) cell line and an adenocarcinoma cervical cancer (HeLa) cell line. Cytotoxicity was measured using XTT (Sodium 3'-[1- (phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro] benzene sulfonic acid hydrate) colorimetric assay, and low cytotoxic effect was exhibited by the extract on the studied cell lines. IC50 on Hela cell line was >200 μg/mL and on Vero cell line was 138.4±2.60 μg/mL. In Poland, Berdowska et al. (2013) evaluated the cytotoxicity of dried aqueous extracts from *Thymus vulgaris L* on two human breast cancer cell lines: Adriamycin-resistant MCF-7/Adr and wild-type MCF-7/wt by the MTT assay, and found that *Thymus vulgaris L* exhibited cytotoxicity against both cell lines with higher toxicity against MCF-7/Adr.

2.6. *In vitro* micropropagation

Micropropagation is a method of plant multiplication from original plant explants. It contributes to the reproduction of high numbers of plants which share the same genes as the main plant thus producing genetically uniform plants. *In vitro* propagation techniques are used to in the production of medicinal and horticulture plants of economic importance (Pati et al., 2006).

Micropropagation is successfully used in the propagation of plants free of fungal and bacterial diseases. These explants are propagated in sterile conditions and if infected could be subjected to several treating protocols during the process. In plants with low reproduction rates, the method meets the required production of such plants. Explants are cultured in *in vitro* conditions where the plant is removed from its original environment and introduced to a sterile and controlled one. Usually placed in petri-dishes, test tubes and other glass material where the explant is settled in a nutrient media. *In vitro* cultivation is not restrained to micropropagation it also includes methods like sanitation of plant material, studying physiological processes, producing secondary metabolites and genetic manipulation (Pavlová, 1992). Micropropagation is a cost-effective way used for commercial plant propagation on several plants species to obtain higher amounts with improved qualities of plant material compared to conventional vegetative propagation (Altman and Loberant, 2010). Different processes for the formation of new shoots can be used for the propagation method like nodal

(meristem) cultures, shoot tip culture, floral meristem shoots, seed culture, somatic embryogenesis, axial buds and adventive organogenesis. When using nodal cultures the intended part to propagate is the meristem and therefore the stem of the plant is cut above the upper and under the lower portions of stem. Propagating shoot tips is rather simple where the tip is cut and planted; usually shoot tips express rapid growth. The following cultures depend on the composition of the medium and depending on species may be influenced by the content of growth regulators in medium (Pavlová, 1992). Plant genotypes have an important role is the effectiveness of *in vitro* propagation and the success of the method. External factors that we are able to control like composition of the nutrient medium, temperature, light intensity and duration, help us to optimize the cultivation and eliminate pathogens (Pavlová, 1992).

2.6.1 *In vitro* micropropagation of *Thymus spp.*

Despite the widespread use of *Thymus spp.* and numerous studies about its medical effectiveness, few studies exist concerning *in vitro* propagation methods of the species. Ozudogru et al. (2011) mentioned that natural *thymus* plants are inadequate in supporting growing market demands of thyme products and created an efficient *in vitro* propagation protocol for *Thymus vulgaris L.* where they supplemented MS medium with different cytokinins and auxins and resulted in genetically stable plants. Medical studies using extracts of *Thymus vulgaris L.* to inhibit human leukemia THP-1 cells concluded that *T. vulgaris L.* is a potential selective cytostatic for the development of anticancer agents (Ayesh, 2014). Some of these few studies are the organogenesis of *Thymus piperella L., Thymus mastichina L.* and *Thymus Boiss.* Or the *in vitro* propagation of *Thymus vulgaris L.* using apical and axillary buds and their multiplication on semi-solid Nitsch and Nitsch medium (Nitsch and Nitsch, 1969; Furmanowa and Olszowska, 1992).

2.7 Polyploidy

The method was discovered in 1907 and the first successful experiment of *in vitro* polyploidization of tobacco was reported by Murashige and Nakano (1966). It is the multiplication of chromosome sets which can occur naturally through abiotic stress, niche differentiation, reproductive isolation or spontaneous mutations and may result with lineage evolution forming new lines of related species (Masterson, 1994). Polyploidy is of relevant

importance in vascular plants, where several plants species have a polyploidy ancestry (Wood, 2009). A large percent of angiosperms ranging from 30 to 80% are polyploids (Bretagnolle, 1995).

Polyploidy is the heritable multiplication of chromosome complements which result in increasing the genome copy number. This process causes morphological changes like the increase of the vegetative parts in the plant, potential increase in number of stomata and amount of chlorophyll by increase in number of chloroplast per leaf. It also leads to phenotypic diversity rapid adaptation. Polyploidy plants are expected to bear more fruit and subsequently have larger fruits (Chloupek, 1992). By increasing genomes via heritable methods, polyploidization plays a big role in plant diversification.

2.7.1 Mitotic polyploidization

Mitotic cell cycles duplicate and distribute chromosomes between two new generation cells. Mitotic polyploidization is achieved by using mitosis spindle inhibitors as colchicines, oryzalin or trifluralin (Wan et al., 1991). Tobacco was the first to be experimented using *in vitro* chromosome doubling (Murashige and Nakano, 1966). Induced polyploidization gained more interest after recent progress in *in vitro* technologies and the induced polyploidy effect of antimitotic agents like colchicine and oryzalin (Wan et al., 1991).

2.7.2 Meiotic polyploidization and cell cytology

Unlike somatic, asexual polyploidization, meiotic sexual polyploidization leads to 2n gamete production thereby being a sexual polyploidization (Ramsey and Schemske, 1998). The union of two 2n or of 1n with 2n gametes leads to the production of polyploidy F2 generations. 2n gametes formation can introduce polyploidy hybrids which express enhanced phenotypic features from their parent diploid cells.

Cytological tools examine cellular conditions where polyploidization changes sexual reproduction in plant cells. Such tools help us understand stability in plant reproduction. Defining cytological mechanisms which concern 2n gamete formation may be further used in agricultural and biotechnological methods and studies like reverse breeding (De Storme, 2013)

2.7.3 Medicinal plant biotechnology

Biotechnology is the science of using the potential of living cells to manufacture products in medicine, agriculture and horticulture. Using modern biotechnologies to select, multiply, preserve, and promote different genotypes of medicinal plants is important for two main reasons. First for its role in conserving the diversity of medicinal plants, an example on this is by using the method of Cryopreservation. Second for meeting the growing demand for herbal medicines or medical products produced from such medicinal plants. 80% of the populations in developing countries and 25% of that in developed countries already rely on such products which are obtained from the wild or cultivated in low amounts (Canter, 2005). Tripathi and Tripathi (2003) stated that *In vitro* regeneration is used to multiply plant cell cultures of medicinal plants and to produce secondary metabolites in several species. Genetic transformation and *in vitro* technologies are relevant biotechnological tools used for the multiplication, genetic transformation and conservation of medicinal plants.

2.7.4 Antimitotic agents

Antimitotic agents are used to induce polyploidy by altering plant cell cycle and inhibiting mitosis. Several antimitotic agents affect cell cycles in different phases. For example colchicine can affect the anaphase cycle where the majority of such inhibitors affect the M-phase mitosis. Colchicine is widely used for its advantage in inducing polyploidy but the compound has several side effects mainly causing mutation in several species. Therefore due to its high affinity in binding to animal tubulins it is toxic to human beings (Morejohn et al., 1987). Alternatives to colchicine include dinitroaniline herbicide called oryzalin (3.5-dinitro-N4, N4-dipropylsulphate). Oryzalin has a high affinity in binding to plant tubulins than to animal once it is affective in inducing polyploidy and is less toxic to animal cells. Several polyploidization experiments were achieved by treatment with oryzalin like Smallanthus sonchifolius (Viehmannová et al., 2009), Lilium (Takamura et al., 2002) and several other species of plants. Although colchicine has its negative effects we cannot determine which antimitotic agent is the most efficient due to different affinities of the agents and different reaction according to plant species (Morejohn et al., 1987).

2.7.5 Methods of detecting polyploidy

Polyploidy can be defined as the possession of three or more complete sets of chromosomes. To detect the number of polyploidy several methods like flow cytometry and chromosome counting can be used. Furthermore since polyploidy chromosomes express different phenotypes from original diploids or haploids we can evaluate morphological or anatomical parameters.

Flow cytometry is commonly used in detecting polyploidy levels. Measuring cell nuclei is released by cutting plant material with sharp razor. Then the nuclei are stained by DNA fluorochrome and passed through the cytometer where a laser based light source illuminates the stained nuclei and detects the light signal in the form of emitted fluorescence (Fig. 3). The DNA analysis is shown on a histogram where the dominant peak belongs to nuclei in G1 phases of cell cycle. The advantage of the method is the rapid testing which examines large samples in short periods of time and provides histograms for several species (Leus et al., 2009). Flow cytometry was used in several researches, e.g. in *Rosa* (Kermani et al., 2003), *Bacopa monnieri* (Escandón et al., 2006), *Punica granatum* (Shao et al., 2003), *Xanthosoma* (Tambong et al., 1998) and other plant species.

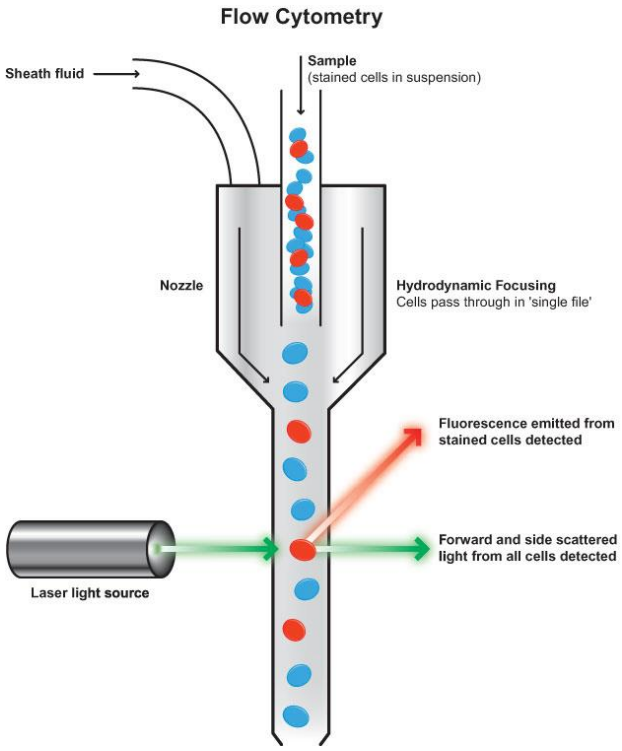

Figure 3: Flow Cytometry

Source: Abcam (2015)

2.7.6 Chromosome doubling in plant breeding

Chromosome doubling can be achieved by the induction of antimitotic agents like trifluralin, colchicine and oryzalin. Factors like plant species, explant type, antimitotic agents, medium and growth regulators used can affect the efficiency of chromosome doubling. After the assessment of type, concentration, and exposure time of the antimitotic agent, explants are tested for determination of efficiency after few multiplication cycles (Dhooghe et al., 2011).

Chromosome doubling results in polyploid plants which express enhanced morphological and anatomic characteristics. These characteristics include an increase of leaf area and thickness, larger flowers and plods and overall darker green color. And enhanced physiological characteristics like disease resistance and higher tolerability against draught stress such physiological upgrades could lead to higher yields with improved qualities of both crop and horticulture plants (Dhooghe et al., 2011).

3. Aims of the Thesis

The main objective of the study was to obtain tetraploid plants (2n=60) chromosome number from diploid plants (2n=30) using *in vitro* induced polyploidization on species of *Thymus vulgaris L.*

Polyploidization could have a positive effect on both morphological and biochemical properties, which can be important in utilization of *Thymus vulgaris L* species as medicinal plants are expected.

Aim of this work was established according to the following assumptions.

H1: *Oryzalin* is an effective antimitotic agent for *polyploidization* of *Thymus vulgaris L.*, respectively of the *Lamiaceae* family

H2: Oryzalin is not an effective antimitotic agent for polyploidization of *Thymus vulgaris L.*, respectively of the *Lamiaceae* family

4. Material and Methods

Polyploidization was carried out in the Laboratory of plant tissue culture in the department of Crop Sciences and Agroforestry in the Faculty of Agriscience in the Czech University of Life Sciences between 2014 and 2015.

Plant material of *Thymus vulgaris L* (2n=30) (Jalas, 1948; Stahl-Biskup and Saez, 2002) was obtained from a specialized horticulture shop. Shoot segments from a single mature plant were sterilized (decontaminated) and then cultivated on MS (Murashige and Skoog, 1962) medium.

4.1 Instrument sterilization

To eliminate or reduce the percentage of contamination certain procedures were maintained. Tools used as petri dishes, scalpels, and tweezers were wrapped in aluminum foil and sterilized for 3 hours at a temperature of 160°C in hot air sterilizer. The operation took place in a flowbox which was sterilized by cleaning with 70% alcohol and then subjected to a minimum of 1 hour UV lamp radiation. After the UV lamp was turned off, the air flow was left to continue the process of air filtering in the machine respectively in the whole working area. Every instrument that was introduced into the flowbox was sprinkled with 70% alcohol. After each step of the propagation process instruments were introduced to 70% alcohol and immediately heated by an ethanol Bunsen burner.

4.2 Preparation of medium

Murashige and Skoog (1962) medium was the only medium used to conduct the following experiment. The content of MS medium is presented in tab. 1. In addition MS medium was supplemented by 30 g/l of sucrose as source of carbon and energy for the explant growth where plants are not yet fully autotrophic. All stock solutions from the table were mixed. 0.1 g/l myo-inositol was added as a carbohydrate necessary to stimulate cell growth and sucrose. Separately 8 g/l agar was added to obtain a rigid structure for the medium. The solution pH was measured by a pH meter. Ascorbic acid and 1M KOH were used to adjust the pH to 5.7. Agar was melted in boiling water and after mixed with the main solution containing the

macro-microelements and vitamins. Lastly, the medium was dispersed into 15 x 2.5 cm test tubes and sterilized in an autoclave for 20 min. under temperature of 121°C.

Table 1. Content of MS medium

Medium Murashige – Skoog			
Stock solutions per 1 liter of distilled water:		Dosage per one liter of stock solution	Per 1 liter of medium (pH 5,7) should be measured:
A	NH_4NO_3	16,5 g	100 ml
	KNO_3	19 g	
	$CaCl_2$	3,3 g	
	$MgSO_4$ x $7H_2O$	3,7 g	
	KH_2PO_4	1,7 g	
B	H_3BO_3	620 mg	10 ml
	$MnSO_4$ x $4H_2O$ (H_2O)	2,23 g (1.69 g)	
	$ZnSO_4$ x $4H_2O$ ($7H_2O$)	860 mg	
C	KI	83 mg	10 ml
	Na_2MoO_4 x $4H_2O$	25 mg	
D	$CuSO_4$ x $5H_2O$	2.5 mg	10 ml
	CoCl x $6H_2O$	2.5 mg	
E	Na_2EDTA	3,72 g	10 ml
	$FeSO_4$ x 7 H_2O	2,78 g	
V	nicotin acid	50 mg	10 ml
	pyridoxin	50 mg	
	thiamin	10 mg	
	glycin	200 mg	

Source: Murashige and Skoog (1962)

4.3 Plant sterilization

Around 8 cm of shoot segments of *Thymus vulgaris L.* were cut clear of roots and leaves. Then washed thoroughly under running distilled water for 1 hour, and soaked in 70% ethanol for 30 s. Finally it was decontaminated with 1 % sodium hypochlorite (NaClO) with two drops of Tween 20 solution for 25 minutes.

After sterilization plant material was moved into the flow box where it was rinsed 3 times in sterile distilled water. Then it was placed on petri dish where a careful process of removing damaged parts and dividing the shoot segments into smaller nodes took place. 4 nodes each about 1 cm in length were replanted into test tubes with MS medium. Material was placed into clima-box under conditions of 16 hours day/8 hours night photoperiod, with 25°C day/23°C night temperature and light intensity of 2500 lux.

4.4 Polyploid Induction

Nodal segments at an average length of 1 cm were cultivated on Ms medium along a period of 60 days. This was done until we reached the number of segments required to proceed with the experiment. As an antimitotic agent the dinitroaniline herbicide called oryzalin was used. Plant material was introduced into a 10mM Stock solution of oryzalin. To prepare the solution we weighed 0.0346 g of oryzalin and dissolved it in 10 ml of dimethylsulfoxide (DMSO) in a sterile flask. Due to sterile function of DMSO we did not have to sterilize the solution.

Nodal segments of *Thymus vulgaris L.* were tested in subsequent oryzalin concentrations: 20, 40, 60 and 80 μM/l during 2 exposure times 24 and 48 hours. 40 nodal segments were treated in each concentration. Nodal explants where inserted in MS medium in and soaked in oryzalin solution for two exposure times, 24 and 48 hours. After the exposure time, nodal segments were removed from the oryzalin solution, rinsed for three times in sterilized distilled water and cultivated individually on MS medium free of growth regulators (Fig. 4). The cultures were maintained at 25/20 ± 0.3 °C under a 16/8 h light mode in cultivation box with (POL-EKO ILW350/350 STD), with 2500 lux light intensity supplied by white fluorescent lamps (Philips LT5 14 W/840). Then the viable explants were multiplicated to the next generation by using nodal segments.

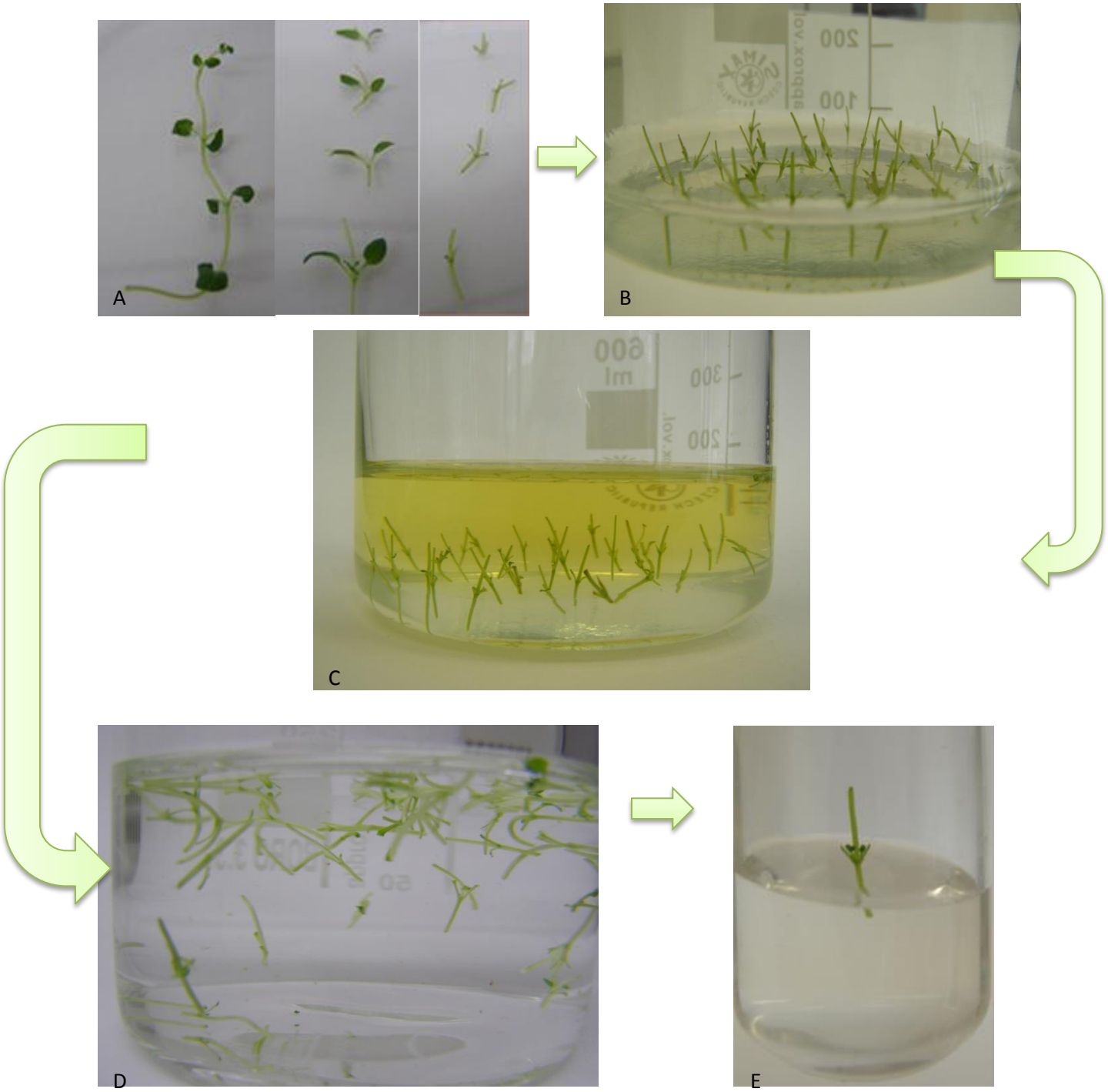

Figure 4. Oryzalin treatment on nodal segments of Thyme; A- nodal segments; B- nodal segments in MS medium, C- nodal segments immersed in oryzalin treatment; D- nodal segments in distilled water; E- nodal segments in test tubes on MS medium

Source: Author, 2015

4.5 Methodology of detection of ploidy level

DNA-ploidy levels were measured using flow cytometry. A technology used to measure and analyzes cells as they flow in a fluid stream through a beam of light. Small parts from leaf tissue were chopped using a razor blade in a Petri dish containing 500 µl of Otto I buffer (0.1 M citric acid, 0.5% Tween 20). Samples of crude suspension containing the isolated nuclei were subsequently filtered through a 50 µm nylon mesh. The second step was to dye the nuclei so a 1 ml of Otto II buffer (0.4 M $Na_2HPO_4.12H_2O$) containing fluorescent dye DAPI (4′.6-diamidino-2-phenylindole) in 2 µg/ml concentration was added to the filtered samples. All measurements to detect ploidy levels were executed in relative fluorescence intensity of at least 3,000 nuclei and was recorded using Partec PAS flow cytometer (Partec GmbH, Münster, Germany) equipped with a high-pressure mercury arc lamp.

5. Results and discussion

Flow cytometry measurement to detect ploidy levels was carried out 2 months after the oryzalin treatment. 60 influenced and 5 control samples were analysed on flow cytometer. Flow cytometric analysis showed the relative DNA content in histogram with a peak corresponding to G0/G1 nuclei of the control diploid plant (Fig. 5)

Out of 60 influenced plants, 3 tetraploid plants were obtained from treatment of 80 μM of oryzalin for 24 h and 1 tetraploid in 48 hours and 1 tetrapoid from treatment of 60 μM of oryzalin for 24 h. Polyploidization efficiency (%) according to the variants was 4.7% (tab. 2).

Table 2. Effect of *in vitro* oryzalin treatment on the survival rate and number of polyploids in *Thymus vulgaris L.*

Oryzalin concentration (μM)	No. of explants (nodal segments)	Treatment duration (h)	Survival rate (%)	No. of tetraploid plants	Polyploidization efficiency
20	40	24	32.5	0	0
	40	48	25	0	0
40	40	24	2.5	0	0
	40	48	25	0	0
60	40	24	12.5	1	2.5
	40	48	5	0	0
80	40	24	32.5	3	7.5
	40	48	15	1	2.5
Total	320			5	4.7

Source: Author (2015)

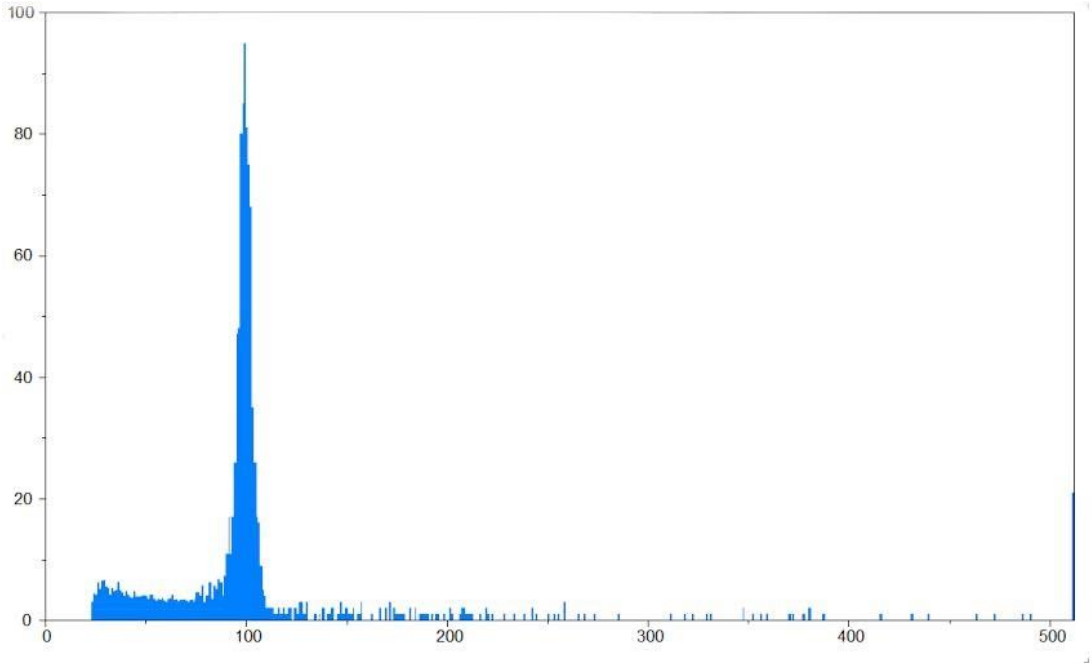

Figure 5. Histogram of relative DNA content with a peak corresponding to G0/G1 nuclei of the control plant (diploid plant)
Source: Author (2015)

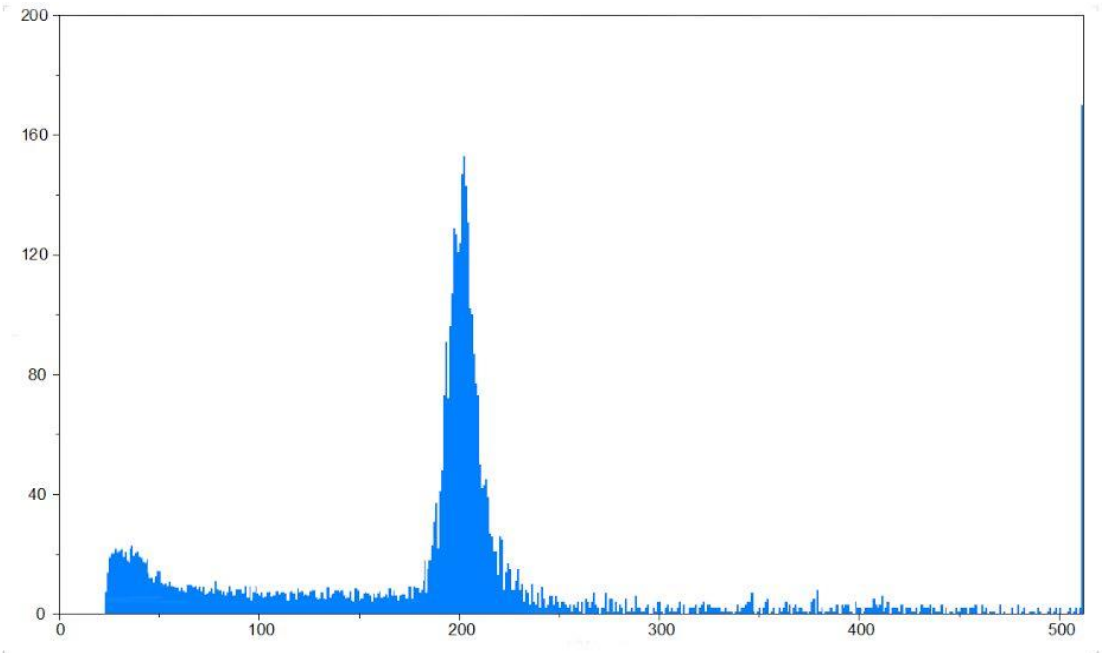

Figure 6. Histogram of relative DNA content with a peak corresponding to G0/G1 nuclei of the plant sample 80µM for 48 hours, (tetraploid) plant on channel 200
Source: Author (2015)

Ploidy level tested by flow cytometry showed the DNA content of each plant. In (Fig.6) tetraploid plant expressed double the number of DNA which control diploid plant expressed in (Fig.5) which identified and confirmed tetraploid plant.

Morphological changes were observed in all tetraploid samples compared to control plants. The sample treated in 80 μM of oryzalin for 24 hours had significantly thicker stems and a darker green color especially in the leaves. There was also an increase in leaf thickness and number per plant compared to the control plant. Another increase, which was in root size and density was observed (Fig. 7).

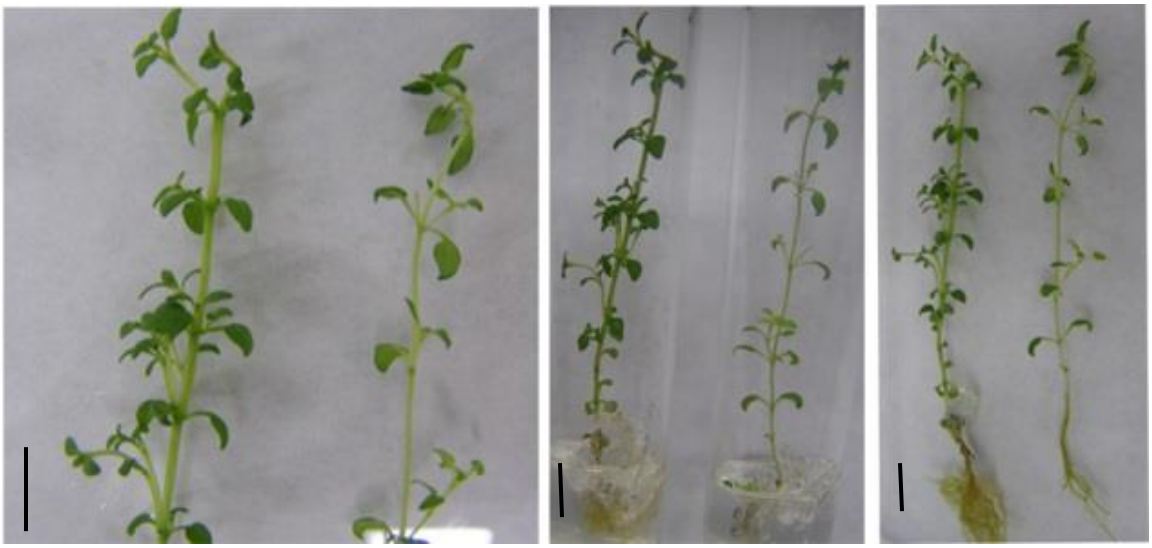

Figure 7. Tetraploid plant (80 μM/24 h) on the left side in comparison with control plant on the right side
Source: Author (2015)

Induction of polyploidy in *Thymus vulgaris L.* using oryzalin is yet to be studied and further experimented. There have been no studies about polyploidization of *Thymus vulgaris L.* Other studies about medicinal plants belonging to the *Lamiaceae* family exist. *In vitro* induction was performed on *Ocimum basilicum L.* a culinary herb from the *Lamiaceae* family

for its essential oils used in dental and oral products as well as in fragrances and in medicine. Omidbaigi et al. (2010) produced autotetraploid plants of basil (*Ocimum basilicum L.*) using colchicine and determined the most efficient treatement at 0.50% concentration. His obtained tetraploids had an increased chloroplast number with larger stomata and pollen grains. The author used several techniques to identify polyploidy levels and stated that flow cytometry was the most effective method in detecting polyploids. This facilitates screening of polyploidy levels at critical experimental experiment steps. We agreed that flow cytometry is a reliable method where we were able to confirm our tetraploid levels in a clear and efficient way in a short period of time. After the confirmation from the flow cytometer we were able to confirm that morphological changes in affected plants were the result of induced polyploidy; tertaploids in our study.

Allium sativum L. a species from the Amaryllidaceae was also treated with a concentration of 0.50% colchicine. Although not from the same family both plants thyme and garlic shared antibacterial and anti-fungal activities. The induction of polyploidy to increase the content of allicin the pharmaceutically active compound in garlic was performed and resulted in tetraploids that possessed thicker and darker-green leaves. These morphological changes between control and treated plants with the antimitotic agent were taken as phenotypic characteristics which expressed genotypic change. The tetraploids obtained had a 30.7% increase in allicin (Dixit and Chaudhary, 2014). We obtained similar morphological changes and flow cytometric results showed tetraploidy levels. So we expect to obtain an increase in compounds such as Thymoquinone (TQ) and phenolic acids like thymol and other essential oils in thyme. Therefore we recommend further study and analysis of the obtained tetraploids from *Thymus vulgaris L.*

We obtained a relatively low polyploidization efficiency that ranged from 2.5 to 7.5 %. Chakraborti et al. (1998) achieved their highest tetraploidy level at a frequency of 39.4 +/- 4.8 % in treating mulberry (*Morus alba L.*) with 0.1 % colchicine. This difference in efficiency rate is due to several reasons. Even though both plants are angiosperms they belong to different families and possess far botanical characteristics. Another reason is due to use of different antimitotic agent where we used oryzalin instead of colchicine. An aspect of the experiment is also different in which we used auxiliary buds where in mulberry apical buds

where used. Finally the medium we used for *Thymus vulgaris L.* was free of plant-growth regulators which may result in mutations, while in *Morus alba L.* the root growth of tetraploids was on basal medium containing 2.6 μM 1-Naphthaleneacetic acid (NAA), a synthetic plant hormone in the auxin family.

In the treatment of *Thymus vulgaris L.* with oryzalin we used four concentrations. Our result showed that polyploidization occurred in variants treated with high concentrations where 80 % of the five polyploidy explants where in concentration of 80 μM oryzalin and the last explant in 60 μM. A higher result of 200-300 μM oryzalin concentration was used in haploid polyploidization on pear (Bouvier et al., 2002). Viehmannová et al. (2009) obtained highest efficiency of tetraploid induction from treatment of *Smallanthus sonchifolius* with 20 and 25 μM oryzalin for 48 h. The highest oryzalin concentration in their study was 30 μM where the lowest in our study was 20 μM of oryzalin. The variation in oryzalin concentration between studies from harmony to disharmony could be related to time in which the experiment was performed in relevance to plants biological hour, parts of plants used, medium and mainly to the plant itself.

6. Conclusions

Using induced polyploidization *in vitro* were obtained tetraploid plants (2n=60) from diploid plants (2n=30) in species *Thymus vulgaris L.* Percent of success was 4.7 %. Plants were obtained in concentrations of 60 μM (1 plant) and 80 μM (4 plants).

Obtained results confirm the first hypothesis, that oryzalin is an effective antimitotic agent for obtaining polyploidy plants using somatic polyploidization *in vitro.*

Obtained polyploidy plants will be further studied on morphological and phytochemical properties.

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