CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Tropical AgriSciences

Department of Crop Sciences and Agroforestry

Induced polyploidy in species from the Lamiaceae family: *Thymus vulgaris* L. and *Satureja montana* L.

DOCTORAL THESIS

Author: Ing. Yamen Homaidan Shmeit

Supervisor: Prof. Dr. Ing. Eloy Fernández Cusimamani

Co-supervisor: Assoc. Prof. Ing. Hynek Roubík, Ph.D.

Prague 2023

Declaration

I declare that I have worked on my dissertation thesis titled " **Induced Polyploidization in species from the Lamiaceae family: *Satureja montana* L. and *Thymus vulgaris* L.**" by myself and I have used only the sources mentioned at the end of the thesis. As the author of the dissertation thesis, I declare that the thesis does not break the copyrights of any third person.

Date and time:

_____

Yamen Homaidan Shmeit

Acknowledgements

I would like to express gratitude to Prof. Dr. Ing. Eloy Fernández Cusimamani for his mentorship, guidance and continuous support throughout my doctoral studies.

I also extend my gratitude to Assoc. Prof. Ing. Hynek Roubík, Ph.D., for his reliable support, motivation and guidance that were pillars during my studies and the writing of this thesis.

Likewise, I extend my sincere gratitude to the Faculty of Tropical AgriSciences for the opportunity to work in the Laboratory of Plant Tissue Cultures and to conduct my studies in the exemplary institution that is the Czech University of Life Sciences Prague.

To my family and friends, I express my profound gratitude for your unwavering patience and support during the highs and lows of my studies and the writing of this thesis. To my father, MuDr. Nabih H. Shmeit, you have been the most unfailing support now and always; I extend special thanks to you for your continuous encouragement throughout my years of study.

Thank you!

Table of Contents

List of articles

Shmeit YH, Fernandez E, Novy P, Kloucek P, Orosz M, Kokoska L. 2020. Autopolyploidy effect on morphological variation and essential oil content in *Thymus vulgaris* L Scientia Horticulturae **263**:109095. Q1, IF = 4.342; point value (10 + 295 × F) = 96.95. Citation indexes: 17. https://doi.org/10.1016/j.scienta.2019.109095.

List of manuscripts

Exploring the Physiological and Anatomical characteristics of novel autotetraploid *Thymus vulgaris* L. variety.

Effect of Synthetic polyploidization on the Phytochemical composition and Biological activities of *Thymus vulgaris* L. essential oil.

List of projects

IGA 2021 Grant. *In vitro* induced polyploidy in *Thymus camphoratus* Hoffmanns. & Link. IGA 20213105, co-investigator (researcher).

IGA 2020 Grant. *In vitro* induced polyploidy in *Celosia argenta* L. IGA 20205004, co-investigator (researcher).

IGA 2019 Grant. Induced mitotic polyploidy *in vitro* in *Senecio clivicolus* Wedd. IGA 20195001, co-investigator (researcher).

IGA 2018 Grant. Breeding of common thyme (*Thymus vulgaris* L.) by mitotic polyploidization *in vitro*. IGA 20185012, co-investigator (researcher).

CIGA 2018 Grant. Induction of *in vitro* polyploidization in selected medicinal andaromatic plants. First place out of 42 accepted projects. CIGA 20182021, co-investigator (researcher).

Conference Contributions

Colaplamed 2021, October 13-15. Ecuador. Virtual conference - Poster presentation - A review about induced polyploidization in the medicinal species of the Lamiaceae family. Book of abstracts. The IX Latin American Congress of Medicinal Plants, p 253.

Tropentag 2021, September 15-17. Germany. Virtual conference - Poster presentation - Genetic and morphological stability of autopolyploid *Thymus vulgaris* L. and changes in its anatomy and physiology. Book of abstracts. Towards shifting paradigms in agriculture for a healthy and sustainable future, ID 359, p 33.

Tropentag 2020, September 9-11. Germany. Virtual conference - Oral presentation - *In vitro* induced polyploidy in *Celosia argentea* var. Plumosa. Book of abstracts. Food and nutrition security and its resilience to global crises. Genetic resources and crop improvement, ID 288, p 36.

Tropentag 2019, September 18-20. Kassel - Germany - Poster presentation - Ethnomedicine in the high lands of Chiapas, Mexico. Book of abstracts. Filling gaps and removing traps for sustainable resources management, ID 354, p 313.

Other publications

Klíma M, Shmeit YH, Kopecký P, Vítámvás P, Kosová K, Prášil IT, Fernández-Cusimamani E. Impact of selected antimitotic substances on doubled haploid and polyploid regeneration in microspore cultures of swede (*Brassica napu*s ssp. *napobrassica* (L.) Hanelt.). Czech Journal of Genetics and Plant Breeding. In Press.

Published book chapters

Klíma M, Melnikovová I, Shmeit YH. 2021. Producción de haploides (Haploid Production). Pages 130-153 in Fernandez E, Sopepi RQ, editors. Tecnologías de cultivo *in vitro* en el mejoramiento de plantas (*In Vitro* Culture Technologies in Plant Breeding). UAGRM - Impresiones. Santa Cruz - Bolivia.

List of tables

Table of figures

Abstract

Garden thyme (*Thymus vulgaris* L.) and winter savory (*Satureja montana* L.) are well known for their aromatic, anti-pathogenic and antioxidant properties. They are also commonly cultivated for ornamental, culinary and medicinal uses. The aim of this research was to induce *in vitro* ploypoidy in diploid plants (2n = 2x = 30) of *T. vulgaris* and *S. montana* to obtain tetraploid plants (2n = 4x = 60) with enhanced morphological traits and an increase in the essential oil content. Nodal segments were treated with four different concentrations of oryzalin (20, 40, 60 and 80 μM) for 24 and 48 h. The ploidy levels of treated and regenerated plants were determined by flow cytometry analyses. The chemical composition of hydro-distilled essential oil was analysed by gas chromatography coupled with a quadrupole time-of-flight mass spectrometer. Oryzalin treatment resulted in three autotetraploid plants of *T. vulgaris* and in six autotetraploid genotypes of *S. montana*. The obtained tetraploid plants presented several morphological changes (increased plant weight, height, leaf length, breadth, and thickness) compared to the diploid plants. In *T. vulgaris*, polyploidization demonstrated an increase in the production of secondary metabolites. The yield of tetraploids essential oil as well as the content of several main components also increased, mainly thymol by 20.79 %. The genetic stability of the new autopolyploid *T. vulgaris* genotype was further assessed after two years of growing in field conditions. Chemical analyses confirmed the superiority of the autotetraploid genotype and its potential as a new thymol chemotype. The obtained genotype of *T. vulgaris* can be implemented as a new variety for the commercial production of garden thyme. Furthermore, the new genotypes of *S. montana* will be analysed for their potential as breeding material.

Keywords: flow cytometry, oryzalin, *Satureja montana*, thymol, *Thymus vulgaris*, polyploidy

Abstrakt

Tymián obecný (*Thymus vulgaris* L.) a saturejka horská (*Satureja montana* L.) jsou dobře známé pro své aromatické, antipatogenní, a antioxidační vlastnosti a běžně se pěstují pro okrasné, kulinářské a léčebné účely. Cílem tohoto výzkumu bylo vyvolat ployploidii u diploidních rostlin (2n = 2x = 30) *T. vulgaris* a *S. montana* za účelem získání tetraploidních rostlin (2n = 4x = 60) se zlepšenými morfologickými znaky a zvýšeným obsahem silic z pomocí *in vitro* indukované polyploidizace. Nodální segmenty byly ošetřeny čtyřmi různými koncentracemi oryzalinu (20, 40, 60 a 80 uM) po dobu 24 a 48 hodin. Hladiny ploidie ošetřených a regenerovaných rostlin byly stanoveny analýzou průtokovou cytometrií. Chemické složení hydrodestilované silice bylo analyzováno plynovou chromatografií se čtyřpólovými systémy hmotnostní spektrometrie s dobou letu. Výsledkem indukce oryzalinem byly tři autotetraploidní rostliny *T. vulgaris* a šest autotetraploidních genotypů *S. montana*. Získané tetraploidní rostliny vykazovaly oproti diploidním rostlinám několik morfologických změn (zvýšená hmotnost rostlin, výška, délka listů, šířka a tloušťka). U *T. vulgaris* prokázala polyploidizace zvýšení produkce sekundárních metabolitů; Tetraploidní výtěžnost silice se zvýšila i obsah několika hlavních složek, především thymolu, o 20,79 %. Genetická stabilita nového autopolyploidního genotypu *T. vulgaris* byla dále potvrzena po dvou letech pěstování v polních podmínkách. Chemická analýza potvrdila nadřazenost autotetraploidního genotypu a jeho potenciál jako nového thymolového chemotypu. Získaný genotyp *T. vulgaris* by mohl být implementován jako nová odrůda pro komerční produkci tymiánu zahradního. Nové genotypy *S. montana* budou analyzovány z hlediska jejich potenciálu jako šlechtitelského materiálu.

Kličova slova: oryzalin, *Satureja montana,* thymol, *Thymus vulgaris*, polyploidie, průtokova cytometrie

Resumé

Le thym des jardins (*Thymus vulgaris* L.) et la sarriette (*Satureja montana* L.) sont bien connus pour leurs propriétés aromatiques, anti-pathogènes et antioxydantes. Ils sont également couramment cultivés pour des fins ornementales, culinaires et médicinales. L'objectif de cette étude est d'induire la polyploïdie-*in vitro* dans *T. vulgaris* et *S. montana* pour obtenir des plantes diploïdes (2n = 2x = 30) avec des traits morphologiques améliorés et une augmentation de la teneur en huile essentielle des plantes tétraploïdes (2n = 4x = 60). Les segments nodaux ont été traités avec quatre concentrations d'oryzaline (20, 40, 60 et 80 μM) pendant 24 et 48 h Les degrés de ploïdie des plantes traitées et régénérées ont été déterminés avec la cytométrie en flux. La composition chimique de l'huile essentielle hydrodistillée a été analysée par chromatographie en phase gazeuse couplée avec un spectromètre de masse en tandem quadripôle-temps de vol. Le traitement à l'oryzaline a produit trois plantes autotétraploïdes de *T. vulgaris* et six génotypes autotétraploïdes de *S. montana*. Les plantes tétraploïdes obtenues ont présenté plusieurs changements morphologiques (augmentation du poids, de la hauteur, de la longueur, de la largeur et de l'épaisseur des feuilles) par rapport aux plantes diploïdes. Pour *T.vulgaris*, la polyploïdie a démontré une augmentation de la production de métabolites secondaires ; le rendement en huile essentielle des tétraploïdes ainsi que la teneur en plusieurs composants ont augmentés (le thymol par 20,79 %). La stabilité génétique du nouveau génotype autopolyploïde de *T. vulgaris* a été évaluée après deux ans de culture dans le champs. L'analyse chimique a confirmé la supériorité du génotype autotétraploïde et son potentiel en tant que nouveau chémotype du thymol. Le génotype obtenu de *T. vulgaris* pourrait être mis en œuvre en tant que nouvelle variété pour la production commerciale du thym de jardin; les nouveaux génotypes de *S. montana* seront analysés pour leur potentiel en tant que matériel de sélection.

Mots clés: cytométrie en flux, oryzaline, *Satureja montana*, thymol, *Thymus vulgaris*, polyploïdie

1. Introduction

Thymus vulgaris L. and *Satureja montana* L. are species of the Lamiaceae family endemic to the west Mediterranean Basin. They are well known as aromatic and culinary herbs, but also have important medicinal properties. Plants from this family produce essential oils rich in monoterpene phenols which are expressed by a wide range of chemo-diversity. The high chemical polymorphism of these plant essential oil profiles provides various biological activities. This has led to their implementation as a biosphere-friendly and biodegradable substance for plant protection in agriculture. Their traditional use in ethnomedicine was followed by pharmaceutical applications. The essential oils of these two plants find a broad spectrum of applications in the cosmetic and food industry, among other miscellaneous uses. The demand for essential oil from medicinal plants has increased in the past decade. This increase was encouraged by recent trends in consumer requirements, ecosystem health awareness and changes in agricultural practices such as integrated plant production and bio-agriculture.

From an economic point of view, the yield of plant biomass and the content and quality of essential oils are crucial. This can be affected by various factors, including nutrition, manure application, water stress, seasonal variation and/or processing. However, genetic predisposition is the basic prerequisite; thus, variety selection and plant breeding are the main factors that influence the quality and yield of essential oils. One of the methods of plant breeding is *in vitro* induced somatic polyploidization. This plant breeding technique can improve the quality and yield of essential oils in plants.

The main objective of this research is to achieve *in vitro* induction of autopolyploid *T. vulgaris* and *S. montna* plants in order to obtain new genotypes with improved horticultural characteristics and higher essential oil yield. Oryzalin was our antimitotic agent of choice, and after developing successful polyploidization protocols, we confirmed the ploidy level of both plant species using flowcytometry analysis. Morphological, biochemical, anatomical and physiological analyses were performed to evaluate the effect of polyploidy on *T. vulgaris* induced genotype.

2. Literature review

2.1. Plant polyploidy

Polyploidy or whole genome duplication is a major force which leads to evolution and speciation. Polyploidy events have been documented in various organisms, including green plants, vertebrates, and fungi (Wendel 2015). Polyploidy is the increase in genome size caused by the inheritance of one or more sets of chromosomes (Otto 2007). Polyploidy has been reported in prokaryotic and eukaryotic organisms and in somatic and germ cells (Van de Peer 2017). Any species that contains more than two complete sets of chromosomes is a polyploid and its ploidy level refers to the number of complete chromosome sets in its nucleus (Ranney 2006). Plant species from all families and across different levels of ploidy prevailed in several climate regions and continue to flourish (Zhang et al. 2019). From these countless polyploid species, here are a few examples according to ascending ploidy levels: Triploid bananas (Musa spp.) (2n = 3x = 33) (Heslop-Harrison & Schwarzacher 2007); Tetraploid *Triticum turgidum* (2n = 4x = 28) (Yadav et al. 2022); Pentaploid blueberry Vaccinium species (2n = 5x = 60) (Redpath et al. 2022); Hexaploid *Camelina alyssum* (hexaploid, 2n = 6x = 40) (Zhang et al. 2022). Moreover, different levels of ploidy exist within the same species; Hexaploid, heptaploid and octoploid *Psidium cattleyanum* cytotypes ($2n = 6x = 66$, $2n = 7x = 77$ and $2n = 8x = 88$) (Machado et al. 2020).

Polyploidization events can be divided into paleo-polyploids and neo-polyploids. Paleo-polyploids, on the first hand, represent ancient genome duplications which occurred throughout species evolution. These polyploid species occurred several millions of years ago. Many paleo-polyploid species have undergone rounds of genome re-diploidization through the reshuffling and rearrangement of multiple sets of parental chromosomes (Otto 2007). Diploidization leads to descendants that behave cytogenetically as normal diploids while harbouring in their genomes the vestigial evidence of past polyploidy events (Wendel 2015). Neo-polyploids on the other hand, are newly formed polyploid species whether the genome duplication occurred naturally or synthetically (Zhang et al. 2019). Polyploidy can naturally arise from somatic mutation due to a disruption in mitosis, resulting in chromosome doubling in meristematic cells giving rise to polyploid shoots. Polyploids can also result from the union of unreduced gametes; eggs and sperm that have not undergone normal meiosis and still have a 2n constitution (Ranney 2006).

Polyploidization is a driving force of organismal evolution, with implications that range from the cell to the ecosystem. It plays an important role in plant speciation, diversification, genome

evolution, adaptive selection, innovation of gene functions and crop domestication (Zhang et al. 2019). Polyploidization does not introduce new genetic material to cells but increases the DNA content by doubling, tripling, quadrupling or further multiplications of chromosome sets. The increased copies of each chromosome greatly enhance genetic diversity. This diversity can then seed additional modifications to gene expression, epigenetics, gene networks, the proteome, cell size and altered responses to stress (Fox et al. 2020). Cellular and organismal polyploidy arise not only in response to environmental stress but also in an adaptation to it (Fox et al. 2020). For example, autotetraploid *Arabidopsis arenosa* populations are distributed across Northern and Central Europe while diploid *A. arenosa* occur in Eastern Europe and the southern Baltic coast (Arnold et al. 2015).

Changes caused by polyploidization start at the cell level and a polyploid with increased genome size will have larger cells. And the changes at the genotypical level of any organism will be expressed in phenotypical changes (Gantait & Mukherjee 2021). Whole genome duplication can result in wide effects on plants, an important phenotypic effect of polyploidy is the 'gigas effect'. This effect is caused by enlarged cell size that results from the increase in the size of the genome per cell (Becker et al. 2022). The specific effects of polyploidy will vary depending on the species in question, the degree of heterozygosity, the level of ploidy and the mechanisms related to gene silencing, gene interactions, gene dose effect and regulation of specific traits and processes (Ranney 2006).

2.1.1. Autopolyploids and allopolyploids

It is important to distinguish between the origins of a polyploid as they generally fall under two main categories. Allopolyploids originate from the hybridization of two distinct species followed by subsequent doubling. On the contrary, autopolyploids possess duplicated sets of chromosomes that originated either from the same or from closely related species, bearing genome complements where all copies of a certain chromosome are closely homologous (Otto 2007; Bomblies et al. 2016). These classifications can be tricky, for instance, allopolyploidy followed by a further round of genome duplication can form polyploids which fall between the two categories and are referred to as auto-allopolyploids (Hegarty & Hiscock 2008).

Fertility is an important factor determined by the origin of polyploid. Allopolyploids are usually fertile as their genomes come from distinct species, in other words they possess two or more dissimilar sets of chromosomes. These chromosomes are sufficiently different and generally do not pair during meiosis (Bomblies et al. 2016). Autopolyploids can result from

spontaneous genome duplication or from the fusion of unreduced (2n) gametes within a single species (or closely related species). An autopolyploid may or may not be fertile due to the presence of multiple homologous chromosomes (Ranney 2006). During meiosis, these multiple homologous chromosomes may result in spurious pairing, unpaired chromosomes and gametes with unbalanced chromosome numbers (Hegarty & Hiscock 2008). Polyploidy is intentionally used to induce sterility. When an autopolyploid is fertile, tetraploids are crossed with diploids to create sterile triploids. The third set of chromosomes in triploids cannot divide evenly and leads to unequal segregation of chromosomes. Polyploidy can also be used to overcome chromosomal sterility, by doubling the chromosomes of a hybrid, fertility can be restored (Ranney 2006). Both autopolyploidy and allopolyploidy represent ends of a continuum of genetic, chromosomal, and phylogenetic divergence of their ancestors (Soltis et al. 2016).

2.1.2. Meiotic polyploidization

Meiosis is a specialized cell division essential for sexual reproduction. It involves a single round of DNA replication followed by two rounds of chromosome division to produce cells with half the number of chromosomes of the mother cell (De Storme & Geelen 2013). In the process of sexual polyploidization, polyploids are generated by the formation and fusion of gametes with somatic chromosome number 2n. That is, pollen or eggs have somatic chromosome number rather than the gametophytic number (Brownfield & Köhler 2011). These unreduced 2n gametes are the main factors for polyploidy induction in nature. In plant breeding, meiotic polyploidization can reduce the breeding process by one generation since 2n gametes can be used immediately in crossing experiments (Dhooghe et al. 2011). Meiotic polyploids combine the genetic effects of different plants of increased ploidy level which plays an important role in crop development (Manzoor et al. 2019). Alterations of the sexual reproduction process in meiotic polyploidization arise through meiotic defects and cytological abnormalities. Different types of cytological abnormalities during meiosis can give rise to 2n gametes and the genetic composition of these gametes is variable (Ramanna & Jacobsen 2003). Sexual polyploidization as compared to asexual would explain better the success of polyploid species in terms of higher fitness and more genetic flexibility (Cigliano et al. 2011).

2.1.3. Mitotic polyploidization

Mitotic polyploidization is based on the double distribution of somatic tissue. This is achieved by interfering with the plant cell cycle, using antimitotic inhibitors (Malmanche et al. 2006). Mitosis is divided into successive series of stages that include prophase, prometaphase,

metaphase, anaphase, and telophase. At the metaphase, inhibition of the chromosome separation process will result in cells with a doubled chromosome complement (Malmanche et al. 2006). The most comprehensive group of antimitotic agents is metaphase inhibitors such as colchicine and oryzalin. They inhibit chromosome separation by associating with tubulin dimers that form the mitotic spindle (Dhooghe et al. 2011). In a normal case scenario, metaphase is reached when all chromosomes display a bipolar attachment and align at the center of the cell. In mitotic polyploidization, antimitotic inhibitors (colchicine, oryzalin), reduce the attachment of new dimers, depolymerizing the microtubule spindle, and interfering with the correct chromosome passage during mitosis (Dhooghe et al. 2011). This interference inhibits chromosome separation resulting in doubled chromosome complements in cells. At the end of mitosis, an autopolyploid is developed (Dhooghe et al. 2011).

2.1.3.1.Mitotic inhibitors

Mitosis-inhibitors include several plant alkaloids, dintroanilines, phosphoroamidates, pyridines, benzamides, benzoic acid, and carbamates (Dhooghe et al. 2011). Colchicine and oryzalin are the most frequently used antimitotic agents to induce polyploidy that in turn results in chromosome doubling (Salma et al. 2017). Colchicine is extracted from *Colchicum autumnale* L. plants and has been widely used in the induction of polyploidy (Dhooghe et al. 2011). Oryzalin is a mitosis-inhibiting herbicide with increasing use in inducing polyploidy. Both colchicine and oryzalin bind to free tubulin dimers and disrupt further microtubule polymerization. However, an important difference lies in their affinity in binding to cells; colchicine has a high affinity in binding to animal cells, while oryzalin has a high affinity in binding to plant cells (Dhooghe et al. 2011). Other benefits of oryzalin also include its less-toxic nature and low cost (Viehmannová et al. 2009). The use of oryzalin for *in vitro* induced polyploidization increased because it is less toxic than colchicine. However, reviewing recently published polyploidization articles shows that colchicine is still prevalent over oryzalin (Table 1.).

2.1.3.2.*In vitro* somatic polyploidization protocol

The polyploidization protocol is extremely important for a successful change in the ploidy level, avoiding the production of mixoploids, chimera tissues and potentially the production of a stable *in vitro* induced polyploid. Advantages of the *in vitro* induction, manifest in our ability to control abiotic growth conditions, eliminate to a certain level any biotic interference and

supply ultimate nutrition for the experimented plants (Dhooghe et al. 2011; Gantait & Mukherjee 2021).

According to Dhooghe et al. (2011) a polyploidization protocol consists of several sub-processes. Starting with the plant material or explant type, antimitotic agents, concentration and exposure time, application method and ending with confirmation technique. Proliferating tissue is ideal for ploidy induction and several explants (germinated seeds, shoot tips, young root tips, scales of seedlings grown in test tubes, adventitious buds, apical buds, plantlets, callus, somatic or zygotic embryos, nodal segments, and tuber segments) must be tested to find the most suitable for each plant species (Fu et al. 2019). Different types of explants hold dissimilar totipotent cells that eventually respond to the antimitotic treatment (Salma et al. 2017). High concentrations of a mitotic agent can increase mortality, and low concentrations are not successful. Choosing the antimitotic agent can start with referring to polyploidization protocols previously used on plants from the same family. Exposure time and concentration play a combined role in ploidy induction (Dhooghe et al. 2011). Antimitotic agent solutions can be added to nodal segments propagated on nutrient media. Other application methods include the incorporation of the antimitotic inhibitor as a media component; or the application of an antimitotic inhibitor to liquid media supplemented with orbital shaking during exposure time. Several *ex vitro* application methods can be used *in vitro,* such as meristems treated with cotton wads embedded in the antimitotic agent or repeated periodic drop of the doubling agent solution on apical meristems (Iannicelli et al. 2020; Gantait & Mukherjee 2021).

2.1.3.3.Indirect and direct ploidy detection methods

Polyploidization is the possession of three or more complete sets of chromosomes, and following the basic genetic fact that genotypical changes are expressed on the phenotypical level. Thus, we can evaluate physiological, morphological, or anatomical parameters in a polyploid and compare it to its progenitor. Such indirect methods of detection include bio-chemical analyses for essential oil content, antioxidative activity, phytohormone levels and main metabolites analyses; cytological observations of pollen size, trichomes, stomata count and density; physiological analyses to determine photosynthesis activity, chloroplast number in the stomata and stomata guard cells, and chlorophyl intensity; and different morphological parameters of the plants whether *in vitro* or *ex vitro* (Pei et al. 2019).

Direct methods of ploidy detection are required as they provide clear undisputed results that determine ploidy level in influenced plants and their progenitors used in polyploidization.

Chromosome counting is a precise and direct method for ploidy detection. The main disadvantages of this method are that it is prone to human error, laborious, and it must include several repetitions (Dhooghe et al. 2011). Nevertheless, this method is still used and usually is accompanied by flow cytometric analysis (Fakhrzad et al. 2023). Flow cytometry is another precise method commonly used in the detection of di-haploids and polyploids. It examines the optical properties of cells in suspension and measures the size of the nuclear genome (Sliwinska 2018). 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 and is still being used as a direct ploidy detection method in several research, e.g., in *Xanthosoma* (Tambong et al. 1998), *Punica granatum* (Shao et al. 2003), *Bacopa monnieri* (Escandón et al. 2006) and *Erysimum cheiri* (L.) Crantz. (Fakhrzad et al. 2023).

2.1.4. Effects of artificial polyploidy on plants

Polyploidization is a breeding method of random probability pattern that may be analysed statistically but may not be pre-predicted. This theory is evident in secondary metabolites production and gene expression related to biosynthetic pathways (Tavan et al. 2022). Whole genome duplication has significant effects on gene expressions of the induced plant. Manipulation of the genome dosage can change gene expression and enzymatic activity in plants period (Niazian & Nalousi 2020). On the first hand, the overexpression of different genes involved in biosynthetic pathways increases the biochemical production in a polyploid plant. On the other hand, when the whole genome is restructured, different duplicated genes are silenced and up or down regulated, which in turn can lead to reduced biochemical production (Niazian & Nalousi 2020). The general advantages of induced autopolyploid cultures are the proliferation of morphological traits and the change in cytological structures, coupled with novel proteomic features and accumulated secondary phyto-pharmaceuticals (Gantait & Mukherjee 2021).

All genetic breeding strategies have been inspired by nature, and the use of polyploidization is no exception. It affects plant genome, phenotype, physiology, and metabolome (Tan et al. 2019). This allows us to obtain new genotypes with improved morphological, physiological and biochemical properties (Salma et al. 2017). *In vitro* induced polyploidy is currently being carried out on several economically significant agricultural, ornamental, and medicinal plants to achieve morphological, physiological and bio-chemical changes (Table 1.). In many experiments, chromosome doubling is a multi-beneficial breeding strategy. A good example of

polyploidization double effect is the increased rhizome size and yield of *Zingiber officinale* accompanied by significantly higher essential oil content (Parasath et al. 2022). Larger ginger rhizomes are more suitable for market parameters and the increased essential oil content adds value to the benefits of this commodity. Since the first successful experiment of *in vitro* polyploidization of tobacco was reported by Murashige & Nakano (1966). The generation of synthetic polyploids has enabled the development of new and improved cultivars. Chromosome doubling, although not always, results in polyploid plants which express various enhanced characteristics. Enhancements can include the content of desired pharma molecules, plant form, flower colour, size and style, fragrance, vase life and prolonged flowering period (Niazian & Nalousi 2020). This breeding strategy brings us a new range of possibilities e.g., increase in quantity and improvement in quality of valuable compounds. Using polyploidization for breeding medicinal plants can be a solution to avoid main problems of their cultivation like overharvesting and habitat loss, which leads to endangering many medicinal species (Iannicelli et al. 2020). Polyploid individuals often outperform their diploid progeny plants in morphological traits, genetic adaptability, and tolerance to environmental stresses, and - a very important feature in aromatic plants - in increasing biomass and the content of bioactive compounds (Iannicelli et al. 2016). For example, doubling of the *Anemone sylvestris* chromosome resulted in tolerance to artificial infection of *Phytophthora plurivora* and has previously shown tolerance to biotic and abiotic stresses (Sediva et al. 2019). The benefit of polyploidization, and its double/multi-effect has been proved in various medicinal plants including, *Matricaria chamomilla* (Sattler et al. 2016), *Dracocephalum moldavica* (Yavari et al. 2011), *Pogostemon cablin* (Wu & Li 2013), and *Tetradenia riparia* (Hannweg et al. 2016b), where the morphological changes were accompanied with increased essential oil yield.

Several polyploidization reviews have been published in the last decade, and the last one from 2021, reviewed *in vitro* polyploid induction articles published up to year 2020 (Manzoor et al. 2019; Iannicelli et al. 2020; Gantait & Mukherjee 2021). Table 1 contains polyploidization publications from 2021, 2022 and 2023. The table focuses on the achieved polyploidization effect, whether negative or positive, according to the author's results as indicated in each publication. Publications are listed from recent years (2021-2023), and in alphabetical order of the species Latin name per year of publication. The publications were researched on Science Direct, Web of Science and Google Scholar.

Table 1. Recent findings of *in vitro* induced polyploidization (2021-2023)

Plant species	Antimitotic agent	Ploidy level	Polyploidization effect	Author
Erysimum cheiri L. Crantz	+ Oryzalin - Colchicine	Diploid to Tetraploid	+ Increase in trichome number, pollen size, stomata size, and number of chloroplasts in the stomata guard cells. + Leaves were bigger (60 %) and purplish, flowering period was prolonged (65 %), and flowers showed greater longevity (41 %), diameter (47 %) and number (78 %). + Increased anthocyanin (56 %) and total soluble solids (140 %) content. + Significantly increase of Ze, SA, JA and ABA. - Lower accumulation of IAA, GA3 and BR. - Decreased stomata density and pollen number. - Shorter internodes and roots.	Fakhrzad et al. 2023
Allium sativum L.	+ Colchicine - Oryzalin	Diploid (2n = 2x = 16) to Tetraploid (2n = 4x = 32)	+ Significant higher content of organosulfur compounds; allicin, diallyl disulfide and diallyl trisulfide. + Content of indole acetic acid and abscisic acid. - Remarkable dwarfness effect; extremely reduced plant height, thickening and broadening of leaves, disappearance of pseudostem, density reduction and augmented width of stomata. - Significantly lower level of phytohormones; indole propionic acid, gibberellin, brassinolide, zeatin, dihydrozeatin and methyl jasmonate.	Wen et al. 2022
Callisia fragrans (Lindl.)	Oryzalin	Diploid (2n = 2x = 12) to Tetraploid (2n = 4x = 24)	+ Leaf and flower size increased significantly and nearly doubled + Significantly higher sodium, iron and calcium content, and the potassium content was increased by 100 %	Beranová et al. 2022
Cerastigma willmottianum Stapf	+ Trifluralin - Colchicine - Pendimethalin	Diploid (2n = 2x = 12) to Tetraploid (2n = 4x = 24)	+ Improved ornamental properties, including greater compactness, larger flowers and heart shaped leaves, and a greater net photosynthetic rate with higher CO2 assimilation efficiency.	Shi et al. 2022

Citrus aurantifolia C. aurantium	Colchicine	Diploid (2n = 2x = 18) to Tetraploid (2n = 4x = 36)	+ Significant values in terms of leaf area, stem diameter, chloroplast count in the guard cells and size of stomata. + Enhancements in leaf chlorophyll and proline contents. + Relative water content and lower electrolyte leakage. - Shorter and made a lower stomatal density	Jokari et al. 2022
Corylus avellana × *Corylus heterophylla*	Colchicine	Diploid (2n = 2x = 22) to Tetraploid (2n = 4x = 44)	+ Thicker and stronger, as well as greener plantlets + Increased stomatal length + Length and width of stomatal guard cells - Remarkably smaller stomatal density - Much shorter plantlets	Zheng et al. 2022
Chrysanthemum morifolium	Colchicine	Pentaploid (2n = 5x = 45) to Decaploid (2n = 10*x* = 90)	+ Superior agronomic characteristics; shorter plant height, thicker stem and root, darker green leaf, larger flower, bigger stoma, and more chloroplasts per guard cell. + Significant improvements on yield and disease resistance + Higher content of chlorogenic acid, luteoloside and 3,5-O-dicaffeoylquinic acid.	Jiang et al. 2022
Galega officinalis L.	Colchicine	Diploid (2n = 2x = 16) to Tetraploid (2n = 4x = 32)	+ Significantly greater contents of total phenols, total flavonoids and galegine. - Decreased shoot length, number of stems, internode length and stomata density.	Khezri et al. 2022
Mentha × *villosa*	Colchicine	Triploid (2n = 3x = 36) to Hexaploid (2n = 6x = 72)	+ Greater stomata size and guard cells' chloroplast number. + Increase in essential oil yield (64 %). + Newly synthesized α-pinene, sabinene, limonene, menthofuran and borneol. + Increase production of 1,8-cineole (53 %) and pulegone (84 %). - Lower stomata density.	Moetamedipoor et al. 2022
Panax vietnamensis Ha et Grushv.	Colchicine	Diploid (2n = 2x = 24) to (2n = 4x = 48)	+ Vigorous growth + Larger stomatal size, denser stomatal chloroplast density - Lower stomatal density	Diem et al. 2022
Pyrus bretschneideri and	+ Colchicine and Pendimethalin	Diploid (2n = 2x) to	+ Desirable traits such as dwarfism and enlarged leaves. + Higher DNA methylation level	Liu et al. 2022

Pyrus betulaefolia		Tetraploid (2n = 4x)		
Rubus sanctus	+ Oryzalin - Colchicine	Diploid (2n = 2x = 14) to Octaploid (2n = 6x = 56)	+/- Affected guard cells; increased the cell size and reduced cell count. + Significantly higher greenness, petiole length and chlorophyll content of leaves.	Sabooni et al. 2022
Salvia multicaulis	Colchicine	Tetraploid (2n = 4x = 28) to Hexaploid (2n = 6x = 42)	+ Significant increased expression of squalene synthase and β-amyrin synthase genes. + Significant increase in oleanolic, rosmarinic and caffeic acid content. - Significant decreased expression of squalene epoxidase, mixed-function amyrin synthase, and lupeol synthase genes. - Reduced content of ursolic acid and betulinic acid.	Tavan et al. 2022
Zingiber officinale Roscoe	Colchicine	Diploid (2n = 2x = 22) to Tetraploid (2n = 2x = 44)	+ More vigour, larger rhizome and higher yield. + significantly higher essential oil content	Parasath et al. 2022
Brassica rapa And *B. oleracea*	Colchicicne	*B. rapa* Diploid (2n = 2x =20) to Tetraploid (2n = 2x = 40) ---------- *B. oleracea* Diploid (2n = 2x = 18) to Tetraploid (2n = 2x = 36)	+ All checked tissues and organs significantly increased. + Genes involved in cell wall biosynthesis were significantly changed. ---------- - All checked tissues and organs either significantly reduced or unchanged.	Harun et al. 2021
Commelina benghalensis L.	Colchicine	Diploid (2n = 2x = 22) to Tetraploid (2n = 4x = 44)	+ Significantly greater size of pollen grains, capsules and seeds.	Shaikh et al. 2021

Ficus Carica L.	Colchicine	Diploid (2n = 2x = 26) to Tetraploid (2n = 4x = 52)	+ Enhanced chlorophyll and phytohormones content. + Enhanced growth, morphological and phytochemical characteristics	Abdoline jad et al. 2021
Fragaria mandshurica And *F. nilgerrensis*	Colchicine	Diploid (2n = 2x) to Tetraploid (2n = 4x)	+ Thicker and stronger morphological characteristics. + *F. nilgerrensis* is more resistant to anthracnose disease. + Flowering period is significantly longer, with an increase in flower quantity.	Du et al. 2021
Fragaria nilgerrensis	Colchicine	Diploid (2n = 2x = 14) to Tetraploid (2n = 4x = 28)	+ Higher cold resistance + Ca2+, penylpropanoid, TFs, ROS scavenging, ABA, soluble sugars act synergistically in cold resistance	Liu et al. 2021a
Gerbera hybrida	Colchicine	Diploid (2n = 2x = 50) to Tetraploid (2n = 4x = 100)	+ Increased flower size (23 %). + Potential tolerance to abiotic stresses. + Good pollen stability. + Increased stomatal size and thicker leaves and broader scape. - Lower stomata density	Bhattarai et al. 2021
Salvia officinalis	Colchicine	Diploid (2n = 2x = 14) to Tetraploid (2n = 4x = 28)	+ Significant increase in Betulinic acid, and expression level of lupeol synthase gene. - Significant decrease in ursolic acid and rosmarinic acid contents.	Tavan et al. 2021

2.1.5. Exploring the stability of *in vitro* somatic autopolyploids

New autopolyploid cells with increased chromosome sets will face challenges of chromosome segregation instability before they persist and thrive. Yant and Bomblies (2021) review the instability challenges that polyploids face, such as reduced fertility and mitotic instability, or the formation of aneuploids in leaf tissues. An important phenomenon to look at here is the reduction division, where somatic polyploid cells undergo meiosis like reduction division (Storchova 2014).

Early measurements for detection of ploidy level can be misleading. Repeating measurements after a period of time is recommended after *in vitro* induced polyploidization. For example, chromosome doubling of mojito mint (*Mentha* × *villosa*), was checked 2 months and 24 months after polyploidy induction. The polyploidization rate of (15.27 %) was observed after two months in *M.* × *villosa*, and after 24 months, some mixoploidy conversion to higher ploidy

level increased the rate to (18.05 %) (Moetamedipoor et al. 2022). According to different studies, the genomic stability of synthetic polyploids is variable. For instance, Julião et al. (2020) confirmed the stability of the ploidy level *of Lippia alba* over five years after induction. Similarly, ploidy level of *Averrhoa carambola* L. polyploid genotypes induced in 2014 was confirmed to be stable in 5-year-old trees growing in field conditions (Hu et al. 2021). While Harbard et al. (2012) reclassified two tetraploid *Acacia mangium* Willd. plants as diploids and two as mixoploid/chimaera. The instability of tetraploids induced by polyploidization can occur immediately after the induction procedure, in the *ex vitro* transfer stage, or while growing in green house or field conditions.

2.2.Aromatic plants of the Lamiaceae family

According to the latest Angiosperms phylogenic classification, the Lamiaceae family belongs to the domain Eukaryot, Kingdom Plantae, Clade; Angiosperms, eudicots, core eudicots, superasterids, asterids, euasterids I. And the order Lamiales (APG IV, 2016). It encompasses a diverse group of woody plants and herbs characterized by their opposite or overhanging leaves (Novák & Skalický 2017). It is a prominent family within the dicotyledons, comprising over 240 genera. Within the Lamiaceae family, numerous species are characterized by their strong aromatic properties, which arise from glandular trichomes categorized as peltate and capitate hairs. These glandular trichomes have been the subject of extensive research due to their role as secretory structures responsible for producing essential oils (Giuliani et al. 2008).

The Lamiaceae family exhibits a diverse range of plant forms, including trees, shrubs, subshrubs, as well as perennial and annual herbs. The stems of Lamiaceae plants are typically quadrangular in shape, and the leaves are arranged oppositely or in a decussate pattern, although whorled arrangement can be rare and alternate arrangement is very uncommon. In terms of leaf characteristics, the leaf blades are usually entire, lack stipules, but they can be toothed or lobed (Harley et al. 2004; Xu & Chang 2017). In the Lamiaceae family, plants typically produce cymes that contain numerous flowers, although in some cases, the inflorescence can be reduced or consist of solitary flowers. The flowers are organized in clusters called verticillasters, which are arranged either on the nodes or in cymes. The flowers are typically hypogynous, and they are usually bisexual, although occasional instances of unisexual flowers or actinomorphic flowers may occur within the family. In the Lamiaceae family, the calyx is typically connate or fused at the base and forms a campanulate (bell-shaped) tubular or urceolate (urn-shaped) structure. The apex of the calyx is usually divided into five lobes, which can be unequal in size. The corollas are also fused or connate at the base and take

on a tubular or campanulate shape. The corolla typically has a four- or five-lobed limb, with many species displaying a two-lipped arrangement. The colours of the corolla can vary, ranging from white and yellow to red, purple, or blue. Regarding the fruits, they are generally composed of four dry nutlets. These nutlets do not contain endosperm. Tuberous roots are rarely found in the Lamiaceae family (Harley et al., 2004; Xu & Chang, 2017).

2.2.1. Secondary metabolites and essential oils

Essential oils are liquids, or semi-liquid extracts from plants which can be obtained through steam distillation. They can be synthesized by all plant organs including flowers, leaves, stems, twigs, seeds, fruits, roots, wood, or bark. Essential oils are limpid and not coloured; they are soluble in organic solvents with a lower density than water and are lipid soluble (Nakatsu et al. 2000). The biological properties of essential oils include enzyme inhibition, allergy reduction, anti-inflammatory, anticarcinogenic, insect repellent, molluscicidal, and antimutagenic activities (Nakatsu et al. 2000).

Most of the species from the Lamiaceae family are used in perfumery, cosmetics, food and pharmaceuticals (Raja 2012; Khoury et al. 2016; Mamadalieva et al. 2017). These species have a wide spectrum of application in modern medicine due to their production of several metabolites including essential oils, tannins, saponins and organic acids. Essential oils of the Lamiaceae family have anti-microbial, anti-fungal and antibacterial specifications (Lugasi et al. 2006; Matkowski & Piotrowska 2006; Sarac & Ugur 2007). In addition, the medicinal and pharmaceutical industries are increasingly utilizing new plants from the Lamiaceae family in new formulas of medical preparations.

Essential oils of Lamiaceae species that have antioxidant and antimicrobial properties have also been important products for ecological and economic development. As such, the production of these essential oils has proven profitable for other sectors including the food industry and agricultural practices as plant protection products (Nieto 2017).

2.2.1.1. Ethnomedicinal use

Ethnomedicine based on herbal medicine has been used historically dating back to 3,000 BC. Traditional medicine is still supported today by the World Health Organization (WHO) because it has been shown to be an integral resource for health in communities with inequities in access to conventional medicine (WHO 2023). Medicinal and aromatic plants are an important component of human healthcare especially for rural communities around the world who tend to rely on forest flora as a source of food and medicine (Okach et al. 2013). World populations

use between 50,000 to 80,000 flowering plants for medicinal purposes. The WHO estimated that 88 % of all countries use medicinal and aromatic plants for their therapeutic effects (WHO, 2023).

Approximately, all members of the Lamiaceae family have ethnomedicinal properties and have been used by various tribal communities (Jafari et al. 2016). Members of this family are known for the presence of aromatic substances such as essential oils. Lamiaceae species have played a critical role in traditional and modern medicine in countries around the world. Plants from the Lamiaceae family have been known for their antimicrobial properties (Salehi et al. 2018). Recent studies have confirmed the therapeutic role of Lamiaceae plants, and the specific bioactive metabolites used for the treatment of viral infections. For example, the use of *Mentha haplocalyx* Briq., *Pogostemon cablin* (Blanco) Benth. and *Scutellaria baicalensis* for the treatment of SARS-CoV infection (Khan et al. 2021). The traditional use of these plant species to support in the cure of various illnesses could be attributed to the presence of phytochemicals such as alkaloids (*Mentha piperita*), sterols (*Hyptis pectinata*), saponins (*Calamintha nepeta* L.), tannins (*Satureja biflora*), terpenoids (*Salvia* sp.) and phenols (*Satureja boissieri*) (Kurkcuoglu et al. 2001; Okach et al. 2013; Topcu & Kusman 2014; Al-Hajri et al. 2022). Some of the properties on these compounds include:

- Alkaloids are known to have analgesic, antispasmodic and antibacterial properties.
- Saponins have expectorant, anticatarrhal, antimicrobial and cough suppressant properties.
- Phenols possess biological activities such as antiinflammatory, antiulcer, antispasmodic, and anticancer properties.
- Tannins include antiinflammatory, regeneration, anticatarrhal, antimicrobial and soothing effects in addition to antiviral, antitumor, antiinflammatory.
- Terpenoids have soothing relief, antimicrobial, carminative effect, and antiseptic properties.
- Sterols have demulcents and antimicrobial effects (Okigbo et al. 2009; Okach et al. 2013).

2.2.1.2.Medicinal use

Plants from the Lamiaceae family have been widely known to demonstrate high potential and minimum side effects compared to the traditionally existing chemical drugs on several diseases such as cardiovascular diseases, coronary heart diseases, congestive heart failures, ischemic

and hemorrhagic brain strokes, angina, myocardial infarctions, development of thrombosis and many others (Redzic 2010; Khoury et al. 2016).

The most important parts of these plants used for medicinal purposes include the leaves, the roots, and the flowers. These medicinal herbs are most used in Asian and African countries such as Iran, Pakistan, India, and South Africa. A review conducted by Niazi et al. (2019) on the medicinal herbs in the Lamiaceae family used to treat arterial hypertension have shown that these herbs have been used universally for treating high blood pressure, and can be considered as alternative agents for this purpose. Examples of medicinal herbs from the Lamiaceae family used to treat high blood pressure include, *Lamium album*, *Tetradenia riparia*, *Salvia africanacaerulea*, *Polygonum orientale*, *Polygonum punctatum* and *Ocimum basilicum* among several other species from this family (Niazi et al. 2019). The main compounds found to have effects on hypertension, along with examples of plant species from the Lamiaceae family in which these compounds occur are; tannins (*Ocimum kilimandscharicum* Baker Ex. Gurke), flavonoids (*Leonotis nepetifolia* (R.Br) Ait. F), alkaloids (*Ocimum obovatum* (E.Mey.Ex. Benth) N.E.Br), as well as terpenoides (*Hoslundia opposita* Vahl) (Okach et al. 2013). Many of these components have been confirmed to have therapeutic effects against many cardiovascular diseases. For instance, flavonoids can be associated with lower coronary heart disease mortality (Peterson et al. 2012). And terpene derivatives can be associated with numerous biological and pharmacological characteristics including anti-inflammatory effects (Sá et al. 2015).

Topcu and Kusman (2014) have assessed the effect of cholinesterase inhibitors, terpenoids and phenolics/flavonoids isolated from natural sources, specifically from Lamiaceae plants as a potential treatment for Alzheimer's disease. Rosmarinic acid, which is one of the most active phytochemical products, found in several Lamiaceae species such as rosemary (*Rosmarinus officinalis*), sage (*Salvia nipponica* var. *formosana*), lemon balm (*Melissa officinalis*), and catmint (*Nepeta* sp.) have strong antioxidant, anti-inflammatory, and neuroprotective effects which can be used in the treatment of neurological disorders such as Alzheimer's disease.

2.2.1.3.Plant protection use

The use of plants as a natural source of plant protection is an old practice dating back to the Roman Empire (Pavela 2007). Today, there is a growing need for environmentally friendly plant protection methods due to the increasing demand for plant consumption arising from new consumer habits. Many herbs, especially the ones including volatile oils can be a natural source

of protection methods through their antimicrobial, antifungal, low toxicity, and biodegradability functions (Abdolahi et al. 2010; Khalid & Jin 2017; Reang et al. 2020).

Essential oils are complex mixtures of terpene compounds including mono- and sesquiterpenes and mono- and sesquiterpenoids. Examples of Lamiaceae plant species containing these compounds include *Lavandula* sp., *Monarda didyma* L., and *Thymus vulgaris* L. (Bakkali et al. 2008; Isman et al. 2018). The chemical composition of essential oils can be affected by different factors, including the species geographical position, the climate conditions it's found in, its harvesting time and its extraction methods (Gachkar et al. 2007; Rahimzadeh et al. 2016). The essential oils found in plants are natural defense arsenals against pathogens and pests (Theis et al., 2003; Tholl 2006).

Essential oils are environmentally safe because of the natural origins of their secondary metabolites and are easily degraded through natural mechanisms; reason why they are used widely in different sectors including agriculture, cosmetics, and other industries (Isman 2006; Pavela et al. 2016; Rotolo et al. 2016; Rotolo et al. 2018).

Many plants from the Lamiaceae family sharing these properties demonstrate a great potential for plant protection usage. Lamiaceae plants that are widely found all over the world can be cheap raw material to produce these essential oils for the control of fungal pathogens. Using these naturally occurring plant products as biofungicides provides an alternative to conventional pesticides (Feng et al. 2011; Mamgain et al. 2013).

Although not completely known, essential oils from Lamicaea species seem to affect pests in different ways. This is due to their differences in structures and physiological and behavioural modes of action of their respective essential oils. Moreover, generally pests tend to be less resistant to the pesticidal effects of these essential oils, which makes them very valuable in pest management strategies (Saad et al. 2019).

Many studies have been conducted to test the efficiency of Lamiaceae plants in fighting pests including microbial and fungal pathogens. A review conducted by Ebadollahi et al. (2020) investigated the pesticidal efficiency of essential oil extract form different genera of the Lamiaceae family such as *Lavandula* L., *Mentha* L., *Melissa* L., *Ocimum* L., *Origanum* L., *Perilla* L., *Phlomis* L., *Rosmarinus* L., *Salvia* L., *Satureja* L., *Teucrium* L. and *Thymus* L. The review also included acute toxicity, repellence, antifeedant activities, and adverse effects on the protein, lipid, and carbohydrate contents, and on the esterase and glutathione S-transferase enzymes. Essential oils from Lamiaceae plants and other components, especially monoterpenoids found in plants like rosemary *(Rosmarinus officinalis* L.), sage (*Salvia*

officinalis L.) and peppermint (*Mentha piperita* L.) seem to be efficient alternatives to synthetic pesticide (Bakkali et al. 2008).

Other studies were conducted to compare the effects of different Lamiaceae species. For instance, Lukošiūtė et al. (2020) compared the effects of lavender (*Lavandula angustifolia*) and thyme (*Thymus vulgaris*) essential oils to suppress the growth of different fungal species (*Alternaria spp., Botrytis spp., and Colletotrichum spp.*) *in vitro*. Results showed that thyme essential oil had a significant effect in suppressing the growth of the fungal pathogens, while lavender had lower efficiency.

Moreover, recent studies on *Rosmarinus officinialis* L. tested its effect for thrips management. The study conducted by Li et al. (2021) investigated the behavioural responses of three thrips species, *Frankliniella occidentalis* (Pergande), *Frankliniella intonsa* (Trybom), and *Thrips palmi Karny* (Thysanoptera: Thripidae) to *Rosmarinus officinalis*. Findings showed that rosemary is a promising repellent plant against thrips pests.

2.2.1.4.Food industry use

The use of aromatic plants as spices is an ancient practice known in all cultures. They are usually used to improve the sensory characteristics of food but also for their nutritional and healthy properties (Nieto 2017). Many herbs can also be excellent substitutes for chemical preservatives. Nowadays with the growing need to develop safer and natural food preservatives, many plants and herbs have gained attention to become alternatives to synthetic additives for the food industry (Pandey et al. 2017). Once again, Lamiaceae family is one of the important plant families to produce essential oils including antioxidants and antimicrobial properties. There is an increased interest in exploring the use of essential oils with antimicrobial and antioxidant properties to increase the shelf life of food. Essential oils extracted from Lamiaceae species such as *Rosmarinus officinalis* and *Origanum vulgare* have shown promising results with respect to their capacity to preserve food (Nieto 2017). For instance, the low risk of resistance and low toxicity for humans which characterizes these essential oils have made them efficient natural products for the preservation of food products such as meat.

Several studies have shown the benefits of using essential oils to preserve meat by extending shelf life, improvement of aroma and taste, and health benefits due to antioxidant and anticancer effects (Alirezalu et al. 2020). However, some disadvantages have been highlighted such as direct contact with essential oils which affects the organoleptic properties of the meat. Moreover, essential oils can cause the elimination of certain types of bacterial populations and

produce favourable environments for the growth of undesirable microorganisms (Hernández et al. 2016).

Other food products such as bread have been tested for the effect of reduced antifungal activities (mainly *Aspergillus niger* and *Penicillium*) through the usage of aromatic plants in dry or essential oil forms. Three plants were selected from the Limiaceae family: *Origanum vulgare* ssp. *hirtum*, *Thymus capitatus* and *Satureja thymbra*. All three plants showed antimicrobial properties and can be considered as a potential source of antimicrobial component for the food industry (Skendi et al. 2020).

2.2.2. Polyploidization effect on Lamiaceae biochemical profiles

Polyploidy can cause both genetic and phenotypic diversity while playing an important role in plants evolution (Xing et al. 2011). Artificial polyploidy is currently used to increase a plant's production potential. The genomic multiplication in polyploid plants may show elevated concentrations of secondary metabolites or the formation of new metabolites compared to their counterparts (Niazian & Nalousi 2020). The idea behind it is that gene duplication results in the increase of genetic resources while hiding deleterious mutations through compensation. In polyploidization, new possibilities in gene evolution are offered. For instance, one copy of the gene can be degraded, while other copies can be conserved, or their expression may diverge (Makova & Li 2003; Innan & Kondrashov 2010; Kondrashov 2012). Therefore, the duplication of many genes can provide extra genetic material and increase genetic variations which is considered an important mechanism in evolution (Kondrashov 2012; Van de Peer 2017). Changes at the genetic level of polyploid plants affect the organization and thus the function of the genome. These changes in the cellular dynamics of polyploid plants can alter the breeding characteristics and aromatic contents. Induced polyploidy influences cell size, organelle size, net photosynthetic rate, elevated metabolic pathways and gene action (Gantait & Mukherjee 2021). Furthermore, chromosome doubling of the genomic content lead to changes in metabolism pathways where enzyme production and protein synthesis are enhanced. This ultimately changes the secondary metabolite biosynthesis (Gantait & Mukherjee 2021). Enhancements in the synthesis of secondary biometabolites may result from changes at the genetic, morphological and physiological levels.

Studies have proven that polyploid plants show specific agronomic characteristics including thicker, rougher and larger leaves, longer and larger stems and roots, larger stomata but fewer in numbers. These changes indicate that polyploidy plants have greater yield than the diploid plants (Gao et al. 2002). This was confirmed in more recent studies; Talei and Fotokian (2020)

demonstrated that physiological changes including changes in the total amount of chlorophyll and secondary metabolites, are the primary results shown through screening of polyploidization breeding programs.

The increase in essential oil content in tetraploid individuals has previously been observed in several Lamiaceae species like *Dracocephalum moldavica*, *Pogostemon cablin*, and *Plectranthus esculentus* (Yavari et al. 2011; Wu & Li 2013; Hannweg et al. 2016a) as well as in other families of aromatic plants (Iannicelli et al. 2016; Tsuro et al. 2016; Noori et al. 2017). Considering the common variability in essential oil composition, even within a single species, it is not surprising that not only the essential oil yield but also its volatile profile and concentration of active constituents is affected by polyploidization (Sattler et al. 2016). An increase in secondary metabolites has frequently been reported (Iannicelli et al. 2020), yet opposite effects have been described in some recent studies.

Urwin (2014) recorded a three-fold increase in essential oil yield for polyploid *Lavandula* × *intermedia* plants. Omidbaigi et al. (2010a) obtained autotetraploid plants from diploid genotype *Ocimum basilicum* L. with a significantly increased content of essential oils. In the C1 generation, tetraploid plants produced 69 % more essential oil than diploid plants.

A study conducted by Hannweg et al. (2016a), where *in vitro* polyploidization was done to evaluate the nutritional value and nematode tolerance of induced tetraploids compared with diploids. Results showed that there was no significant difference in mineral contents in both leaves and tubers for tetraploids and diploids. However, more tetraploids revealed better tolerance to rootknot nematode.

Chinese sage *Salvia miltiorrhiza* Bunge has been known for over two thousand years to be a very efficient plant used in Chinese medicine. The plant has been studied over the years to determine the efficiency of induced polyploidization for commercial usage. Gao et al. (1996) conducted polyploidy induction on *Salvia miltiorrhiza in vitro*, the specimens were transferred to the field to screen for more than 15 agronomic characteristics of these plants. Most of the tetraploid plants grew dynamically and showed bigger stomata and larger stems and roots.

The tetraploid plants also turned to be semi sterile. According to Gao et al. (1996), the study discussed several advantages growing polyploidy plants by tissue culture; (1) through tissue culture, a larger number of buds, seed, or seedling can be treated effectively. (2) It is more convenient to determine chromosome numbers in the roots of polyploid plants *in vitro* in comparison with those of field plants. (3) Material selected from polyploidy plants may be rapidly propagated by tissue culture for further field identification and commercial production.

Twenty-two years later, studies on *Salvia miltiorrhiza* continued where Chen et al. (2018) obtained tetraploid plants with enhanced agronomic traits. Moreover, concentrations of dihydrotanshinone I and total tanshinones in the root extract of the tetraploid plants showed to be significantly higher when compared with the diploids. Finally, the tetraploid plants also showed to be stable and displayed consistent ploidy levels for 10 generations of plantlets and seedlings (Chen et al. 2018).

Another plant from the Lamiacea family known for both its medicinal and culinary usage is *Salvia officinalis* L. The plant has also been extensively studied to investigate its polyploidy inductions for commercial usage. One of the most recent studies conducted by Hassanzadeh et al. (2020) attempted to show the effect of polyploidy induction in *Salvia officinalis* through seed treatment with different colchicine concentrations and durations. Results showed that survival rate decreased with the increase of treatment time and concentration. As mentioned above, preliminary results of induced tetraploidy showed changes in morphological, anatomical and physiological characteristics. Indeed, tetraploid plants presented larger leaf length, leaf width, plant height, number of leaves, number of nodes and internodes length. Tavan et al. (2021) studied the effects of both *in vitro* and *in vivo* induced polyploidization on morphological characteristics, anatomical structures, phytochemical traits, and expression level of the genes in *Salvia officinalis*. Results confirmed differences in polyploids leaf shape and colour, leaf and stem thickness, trichrome density, root length, plant height and number of leaves. While the current study indicated changes in rosmarinic acid content in polyploidy plants, it also indicated an increase in squalene epoxidase (SQE) and lupeol synthase (LUS). Thus, induced polyploidy can randomly change the breeding traits and aromatic content of the plants (Tavan et al. 2015).

2.3.*Thymus vulgaris* L.

Thymus vulgaris L., commonly known as garden thyme or common thyme, is a perennial aromatic plant frequently cultivated for its ornamental, culinary and medicinal uses. It is the most commercially cultivated species in the genus *Thymus* (Stahl-Biskup & Sáez 2002). The basic number of x = 15 and 2n = 30 is determined for diploid *T. vulgaris* according to the Index to Plant Chromosome Numbers (Novak & Blüthner 2020) and the Chromosome Counts Database (Rice et al. 2014). Dietary trends in recent decades increased the demand for culinary herbs (Rowland et al. 2018). Because of their anti-pathogenic and antioxidant properties, thyme essential oil is being intensively used in several fields—mainly in the medication, agriculture,

and food industries. In the European Union, the cultivation area of aromatic, medicinal and culinary plants increased from 176 in 2015 to 220 thousand hectares in 2018 (Eurostat 2019). An estimated 4 tonnes of *T. vulgaris* essential oil is produced each year, while over 15,000 tonnes of *Thymus* sp. are traded globally each year (Novak & Blüthner 2020).

2.3.1. Botanical classification and description

According to the Angiosperm Phylogeny Group's arrangement to date, the botanical classification the species *T. vulgaris*, falls under the Family Lamiaceae, Subfamily Nepetoideae, Tribe, Mentheae, Subtribe Menthinae, and the Genus Thymus (Lamiaceae) (APG IV, 2016).

T. vulgaris is a perennial evergreen herbaceous and woody subshrub growing from 10 to 30 cm in height. Like other Lamiaceae species, *T. vulgaris* plants have trichomes and aromatic glands containing essential oils (Fig. 1). These trichomes form at a very early stage during the leaf development and contain volatile essential oils which are responsible for the pungent aroma characterized with *T. vulgaris* (Fig. 2).

Figure 1. *Thymus vulgaris* aerial parts (Author)

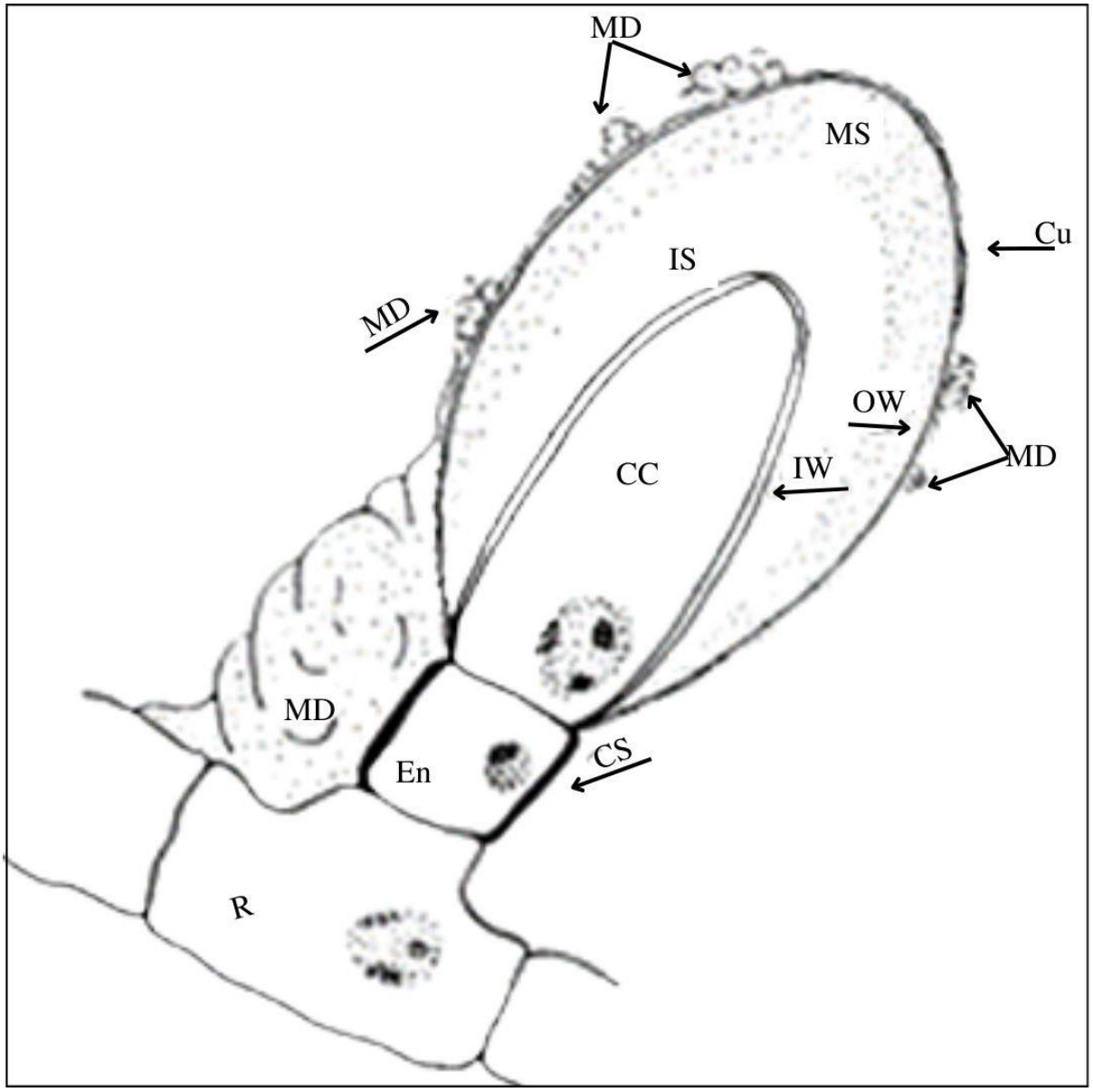

Figure 2. Longitudinal section of a mature mucilage producing trichome of *Thymus vulgaris* (Modenesi et al. 1984).

2.3.2. Origin and geographical distribution

Thymus vulgaris L., is an aromatic and medicinal plant from the Lamiaceae family endemic to the west Mediterranean Basin. Growing naturally in the Iberian Peninsula and in Northwest Africa, and commonly cultivated from Spain to Italy. Its cultivation area expanded over time to mild–temperate and subtropical climates (Stahl-Biskup & Venskutonis 2012). Today the plant grows spontaneously in different ecosystems. This spread into new habitats is related to early interests in its features which go back to ancient history.

2.3.3. Reproduction and cultivation

The plant is the most studied species in the genus Thymus, and it is cultivated for ornamental, culinary and medicinal purposes. Agronomical studies aiming at increasing essential oil quantity and enhancing its quality dominate the research (Trindade et al. 2018). Regarding cultivation, vegetative propagation of medicinal and aromatic plants is preferable to seed propagation which has low germination levels (Nicola et al. 2004). As for thyme, seed germination is accelerated by sunlight, and seeds grow within 1 to 2 weeks between 10 and 30 °C. In warm climates, the favourable period for seed propagation is around March at the end of winter. But while thyme can be propagated from seeds, its germination vitality is relatively low (50 to 72 %) (Nicola et al. 2004). After three to four years of growth, when stems become woody and the plant has fewer leaves, *T. vulgaris* is separated using root division and replanted (Nabavi et al. 2015). And since it is easily propagated from cuttings, vegetative propagation prevails (Iapichino et al. 2006).

The growth rate, morphology and phytochemical profile of thyme varies between geographical regions and seasonal variations (Neocleous & Ntatsi 2018; Lemos et al. 2017). Moreover, the biomass yield and essential oil character are affected by different agrotechnical factors like, water stress and manure application (Askary et al. 2018), bio-compost substrate (Bolechowski et al. 2015), foliar nutrition (Pavela et al. 2018) and planting space and harvest time (Al-Ramamneh 2009; Badi et al. 2004). It is notable to mention here that genetic predisposition is the basic prerequisite; thus, variety selection and plant breeding, are other factors that influence the quality and increase the yield of essential oils (Bhardwaj et al. 2020; Shmeit et al. 2020).

2.3.4. Biochemical properties

In nature, essential oils play an important role in plant protection as antiviral, antibacterial, antimycotic, insecticide and against herbivores (Bakkali et al. 2008). These effects are attributed to phenolic acids and other phenols and especially to the essential oil content. In thyme, this consists mainly of thymol, carvacrol, geraniol, α-terpineol, 4-thujanol, linalool, 1,8-cineole, myrcene, γ-terpinene, and p-cymene. The abundance of the major components depends greatly on the plant chemotype, while the thymol chemotype is considered the most widespread (Mandal & DebMandal 2016; Trindade et al. 2018).

Thanks to these properties, various plant essential oils can be used as a therapeutic or adjuvant agents in the pharmaceutical industry (Edris 2007; Kokoska et al. 2019), as aromas, flavourings, and natural preservatives in the food industry (Mandal & DebMandal 2016;

Pandey et al. 2017). Finally, they can also be used as biosphere-friendly and biodegradable substances for plant protection in agriculture (Pavela & Benelli 2016). Moreover, *T. vulgaris* and thymol are very promising agents in the food industry for preserving product quality (Sellamuthu et al. 2013; Park et al. 2017).

2.3.5. Ethnomedicinal use

Earliest records of using medicinal plants endemic to the Mediterranean region date thousands of years ago. Evidence of such medical use of a plant in this region comes from the late Minoic Gazi in Crete (1400-1200 BC), in the form of a clay sculpture (Leonti et al. 2009). In *Naturalis Historia*, book 21, Gaius Plinius Secundus (first century AD), describes the abundance of thyme in Narbonne province (southern France), and discusses the therapeutic attributes of thyme varieties (Stahl-Biskup & Sáez 2002). Different *Thymus* spp. are used for culinary purposes due to the pungent taste and attractive odor of these plants. *T. vulgaris* is well known as a culinary herb but it also has documented medical applications. It is traditionally used for the treatment of various complaints of respiratory tract and digestive system issues, and for its antimicrobial effects, among others (Salehi et al. 2018). Going back to the Egyptian civilization, where *T. vulgaris* was used in unguents for embalming practices (Napoli et al. 2010), until recent ethnomedicinal studies documenting the medicinal versatility of this plant. For example, the therapeutic use of an infusion prepared from *T. vulgaris* aerial parts by breast cancer patients in Algeria (Taïbi et al. 2020). The oral administration of *T. vulgaris* by pregnant Palestinian woman for several therapeutic purposes (Al-Ramahi et al. 2013). The use of *T. vulgaris* tea for stomach disorders in south-eastern Serbia (Jarić et al. 2015) or the medicinal use of *T. vulgaris* infusion in Spain (Rivera et al. 2019).

2.3.6. Medical properties

According to EU standards, committee on Herbal Medicinal Products of the European medicines agency, the pharmaceutical use of *T. vulgaris* essential oil is in liquid herbal form for oral use as a cough medicine (*Thymi aetheroleum*), for urinary tract and genital disorders (*Thymi herba*), and in liquid or semi-solid dose forms for cutaneous use and usage as a bath additive (EMA 2020). The European Pharmacopoeia further describes Thyme herb liquid extract from *T. vulgaris* L. as the active component of the Medithyme cough syrup where the effectiveness of thyme as a therapeutic is based on 'traditional use', and on evidence that it has been used safely for at least 30 years including at least 15 years within the EU (HPRA 2021).

.

T. vulgaris is distinguished from other Lamiaceae species by high carvacrol and thymol contents. The thymol chemotype of *T. vulgaris* is widely used where thymol, as the main active compound, exerts antimicrobial, anti-inflammatory, antioxidant, and immunomodulatory activity, and is effective against various disorders of the respiratory, cardiovascular, and nervous systems, among others (Marchese et al. 2016; Salehi et al. 2018). Therefore, its practical use is common in the pharmaceutical industries (Edris 2007; Kokoska et al. 2019). Its pharmacological applications continue to be investigated and reported. The antitumor activity of *T. vulgaris* and its mechanism of action against different human cancer cells is a major medicinal aspect. Thyme essential oils exhibit cytotoxic activities against different cancerous cell lines; P815 mastocytoma cell line (Jaafari et al. 2007), HNSCC head and neck squamous cell carcinoma cell line (Sertel et al. 2011) and other PC-3, A-549 and MCF-7 cancer cells (Zu et al. 2010). Essential oils versus synthetic chemicals prevail in low toxicity and side effect range. Furthermore, *T. vulgaris* essential oils may show proliferative effects on normal human cells while being cytotoxic against cancerous cells; case study of carvacrol cytotoxic effect against P815 mastocytoma cell line and proliferative effect against PBMC normal human peripheral blood mononuclear cells (Jaafari et al. 2007).

2.3.7. Application in plant protection

Because of its versatile biochemical profile, essential oil of thyme can be used as biosphere-friendly and biodegradable substance for plant protection in agriculture (Pavela & Benelli 2016). *T. vulgaris* and its main component thymol are very promising agents for the protection of agriculture crops and stored agricultural commodities (Sellamuthu et al. 2013; Park et al. 2017).

Several *in vitro*, field, and post-harvest trials were conducted in the last 10 years regarding the biological effect of *T. vulgaris* and its monoterpene thymol on different plant pathogens, pests, and weeds. Bacterial blight in soybean caused by *Pseudomonas savastanoi* is combated using synthetic pesticides which is effective but produces bacterial resistance. The diverse biochemical profile of *T. vulgaris* prevents such resistance from evolving. Sotelo et al. (2021) showed that thyme essential oil reduces bacterial growth and the number of cells of the phytopathogenic strains on the surface of soybeans.

Fungal diseases of cereals present a major concern for food security and human health. Bio-pesticides are necessary where synthetic chemical use is limited for integrated crop production and completely prohibited in bio-agriculture. Zabka et al. (2009) tested the antifungal effect of 25 medicinal plants against *Fusarium oxysporum*, *Fusarium verticillioides*, *Penicillium*

expansum, *Penicillium brevicompactum*, *Aspergillus flavus* and *Aspergillus fumigatus*. *T. vulgaris* was determined as one of the most effective against growth of target fungal species. In another study, *T. vulgaris* prevailed among five essential oil baring plants, showing the best antifungal activity against major cereal pathogens including *Oculimacula yallundae*, *Microdochium nivale*, *Zymoseptoria tritici*, *Pyrenophora teres* and *Fusarium culmorum* (Matusinsky et al. 2015).

Pests present another sector of plant protection where using synthetic active compounds leads to tolerance followed by complete resistance and leaves us dealing with different chemical residual effects. Furthermore, broad spectrum insecticides damage the whole ecosystem including beneficial insects. Incomes the benefits of natural essential oil combatants with versatile bio-profiles and specific minimal effective concentrations. Essential oils of *Carum carvi* and *Thymus vulgaris* were efficient causing high mortality of *Meligethes aeneus* adults (Pavela 2011). This pollen beetle is the most important pest of *Brassica napus* L. Essential oils from *T. vulgaris* have strong potential in the production of new insecticides which are safe for the environment and for human health.

Larvicidal activities of four *Thymus vulgaris* chemotypes varied in their biological activity against *Culex quinquefasciatus* mosquitoes. Genetically distinct chemotypes of *T. vulgaris* can be distinguished based on the dominant monoterpene produced in glandular trichomes on the surface of the leaves. The highest larvicidal efficiency here was determined for the Thymol chemotype (Pavela et al. 2009).

Inhibitory, preventive, repellent, mitigative and eradicative effects of *T. vulgaris* extracts as natural fungicides, bactericides, insecticides, nematicides, rodenticides and herbicides are reported in different studies (Catani et al. 2022).

2.3.8. Application in the food industry

Essential oil extracts from *T. vulgaris* plants find a broad spectrum of applications like prolonging the shelf life of several commodities (Mandal & DebMandal 2016; Pandey et al. 2017). Similar to its application as an agrochemical, the general antipathogenic and pest repellent characteristics of *T. vulgaris* essential oils are employed in the food industry. Pinto et al. (2021) reports how essential oils of *T. vulgaris* control *Penicillium* spp. contamination of oranges and extend the shelf-life of citrus fruit. Thyme oil mainly reduces mycelial colonization of the fruit skin and, as a secondary effect it delays the production of new spores. Thus, prolonging storage by preventing further contamination and delaying the source of infection.

Prolonging shelf life is of key importance for commodities with reduced shelf life. Sweet cherry is such an example where preserving the physical and chemical quality in postharvest storage is required. *T. vulgaris* used as an edible coating, delays ripening processes, increases storage time and improves overall antioxidant activity of sweet cherries (Afonso et al. 2023). Strawberry is another such commodity as it is susceptible to decay by fungi. *T. vulgaris* shows high antifungal activity against *Botrytis cinerea*. Its essential oils reduce microbial load and decay. Postharvest losses decreased and thyme oil further preserved the physical and chemical quality attributes of strawberry (Javanmardi et al. 2023). Minimal effective concentrations of thyme oil are not phytotoxic and don't affect sensory parameters.

Food borne pathogens like *Staphylococcus aureus*, *Listeria* sp., *Escherichia coli* and *Salmonella* sp. cause food borne diseases and are a major public health concern. In particular, thyme essential oil excels in inhibiting food pathogens (de Almeida et al. 2023). Thyme oil can be bacteriostatic and/or bactericidal in different concentrations and encapsulating oils influence the antimicrobial effect.

2.4. *Satureja montana* L.

Winter savory or mountain savory, scientifically known as *Satureja montana*, belongs to the Family Lamiaceae, Subfamily Nepetoideae, Tribe Mentheae, and the Genus Satureja (APG IV, 2016). This genus comprises around 200 species of aromatic shrubs or herbs. The distribution of the Satureja genus is widespread, with species found across various regions, including the Mediterranean, Asia, Europe, and certain parts of America (Chorianopoulos et al. 2004). The basic number of x = 15 and 2n = 30 is determined for diploid *S. montana* according to karyological studies (Boscaiu et al. 2000; Shariat et al. 2013) and to the Chromosome Counts Database (Rice et al. 2014).

Species from the Nepetoideae subfamily are known for their high content in essential oils (more than 0.5 % of essential oils) (El-Gazzar & Watson 1970). *S. montana* is a perennial herb native to arid, sunny and rocky regions in the Mediterranean area of Southern Europe and in Northern Africa (Kermer et al. 2015). The species is recognized for its medicinal purposes (Elgndi et al. 2017). Like many other species from the Lamiacea family, *S. montana* provides essential oils that have a wide range of antiseptic, antioxidant, antifungal, carminative and digestive properties. It can be also used for cooking and food preservation (Souto-Maior et al. 2017). A single harvest at the beginning of flowering determine good herb yield from one-year-old *S. montana* plant grown in temperate climate zone and harvested at flowering in a single harvest pattern can yield an average of 90 kg x 100 m^{-2} of fresh herb containing an average oil yield of

1.69% of the raw material (Zawiślak & Nurzyńska-Wierdak 2017).The production of *S. montana* is somewhat variable between countries yet it does not come close to the production of *Salvia officinalis, T. vulgaris* and other medicinal and aromatic plants. Albania for example, annually plants around 5,600 ha of medicinal herbs and is a main exporter to Europe and the United States. In the Albanian region of Shkoder, *S. officinalis* leads the production of around 3,500 ha while *S. montana* occupies the lowest production area of 20 ha (Ibraliu & Meco 2022).

2.4.1. Botanical classification and description

S. montana, is a small shrub characterized by its straightened and ligneous stems. The leaves of this plant are arranged in an opposite or alternate pattern and are composite. The individual leaves are linear-lanceolate in shape, measuring around 26 mm in length and 6.5 mm in width. These leaves have a short petiole, entire margins, and exhibit a leathery surface (Fig. 3). The flowers of *S. montana* can be white or light pink and are arranged in verticillasters in the axils of the bracts, forming bouquets of two to three flowers. Each flower consists of four stamens, with the sepals measuring approximately 4.5 to 6.5 mm in length. The corolla is around 7.5 to 12 mm long. The bloom period for *S. montana* occurs from June to August, and after flowering, the fruit develops as a quadrangular achene. The seeds in the fruit come in brown to black colouration, are small and round, and measure about 0.5 to 1 cm in diameter (Kokkini et al. 2003; Zelený 2012; Matthias & Laisné 2017).

Figure 3. *Satureja montana* aerial parts (Zelený 2012)

2.4.2. Origin and geographical distribution

Satureja montana is a perennial aromatic shrub that is found in rocky mountain ranges of the Mediterranean and Adriatic areas. The wide range of distribution of the species has made it adapted to different microclimatic and edaphic conditions. In addition, the distribution limit of the species with other species from the same genus has led to a high variability in its botanical and chemical characteristics (Aćimović et al. 2022). *S. montana* is a valuable aromatic plant widely spread in the Balkan region (Šojić et al. 2019). It is native to the Mediterranean region but can also be found in Europe, including the Adriatic area, the Dinaric Alps, and the Apennine Mountains in Italy (Di Pietro 2011; Tomaselli et al. 2021). It is also found in Slovenia, Croatia, Bosnia and Herzegovina, Serbia, Montenegro, and Albania. *S. montana* has also been recorded to occur in the Pyrenees of France and Spain (Bezbradica et al. 2005; Redzic 2010; Lumpert et al. 2017; Bojovi´c et al. 2018; Navarro-Rocha et al. 2020).

2.4.3. Reproduction and cultivation

Satureja montana is an allogamous species which means that its reproduction occurs through crosses among plants. The reproductive organs are covered by an indumentum containing both

non-glandular and glandular trichomes. The essential oils found in the glandular trichomes play a role in attracting and guiding pollinators. The type of gland found on *S. montana* is a one basal epidermal cell, a stalk cell, and a head of twelve secretory cells (Marin et al. 2012).

Vegetative propagation is best made through cuttings, layering and division. Since the production of cutting by rooting segments of lignified shoots is expensive, it is recommended to propagate *S. montana* by seeds. A more uniform propagation material with better morphological parameters was produced using generative propagation relative to vegetative propagation (Zawiślak & Nurzyńska-Wierdak 2017).

2.4.4. Biochemical properties

Like other plants from the Lamiaceae family, the genus *Satureja* is characterized by strong odors that come from essential oils. These oils accumulate in glandular trichomes on the surface of stems, leaves, calyces, and corollas (Dodoš et al. 2021). Plants of the genus *Satureja* provide a high variability of chemical compounds even among plants of single species. Their variability depends on chemotype, and the genus has highly complex chemical polymorphism (Ćavar et al. 2013). Nevertheless, the variability does not affect antibacterial, antiviral, fungicidal and antioxidant proprieties of the plant (Santos et al. 2019). Mihajilov-Krstev et al. (2013) studied the changes in chemical composition of essential oils from three *S. montana* subspecies. Results indicated changes in compositions with the change in altitudes. For instance, with the increase in altitude, there was a higher content of linalool, terpinen-4-ol and cis-sabinene hydrate, while the content of phenolic compounds, thymol and carvacrol was lower. These changes had effects on the antimicrobial characteristics of the extracted essential oils. Thus, the essential oils having higher concentrations of phenols and alcohols seemed to have higher antimicrobial potential.

Species from the *Satureja* genus are known for having essential oils with different biological, ecological, and physiological functions. Satureja subspecies can present many differences in their chemical compositions of essential oils (Slavkovska et al. 2001). Many studies have focused on the chemical composition of Satureja spp., revealing the presence of various important compounds. These studies have shown that Satureja species contain volatile oils, tannins, phenolic compounds, sterols, acids, mucilage, and pyrocatechol. Essential oils are mainly composed of phenols, monoterpenes, sesquiterpenes, phenolic acids, and flavonoids (De Rojas et al. 1996).

The quality and quantity of the essential oils in *S. montana* can vary depending on various factors, with the stage of plant growth being a significant influence (Mirjana & Nada 2004).

Thymol, carvacrol, and p-cymene are major components of *S. montana* essential oils. Other essential oil components include linalool, borneol, and geraniol (Santos et al. 2019).

Caprioli et al. (2019) explored some of the major differences in essential oil composition and antioxidant activity between two *S. montana* subspecies; *S. montana* subsp. *variegata* and subsp. *montana*. Main results did not show difference between the two subspecies. In fact, the essential oils analysed through hydrodistillation of flowering parts by gas chromatography/mass spectrometry (GC/MS) showed the presence of carvacrol (22.5 %), p-cymene (17.6 %), thymol (17,4 %), carvacrol (61.9 %), p-cymene (9,9 %) and c-terpinene (8.2 %) (Caprioli et al. 2019).

In another study, López-Cobo et al. (2015) analysed the essential oil of *S. montana* subsp. *kitaibelii*, for the first time by HPLC–DAD–ESI–TOF–MS which is a high-performance liquid chromatography–diode array detector–electrospray ionization–time of flight–mass spectroscopy. The study was able to identify 42 compounds including phenolic acids and flavonoids, among others.

Following that, Zeljkovic et al. (2015) studied the antioxidant activity and phenolic and flavonoids extracts of *S. montana*. The study also aimed at chemically modifying lipophilicity, isolated extracts which were also tested. Results indicated that methanol extracts contained more phenolic compounds compared to chloroform extracts. While chemically modified extracts showed higher amounts of antioxidants as well as better lipophilicity. These results indicate that *S. montana* plants can be used as natural antioxidants of oil, fats and other lipophilic foods.

2.4.5. Ethnomedicinal use

Satureja species have been continuously applied in ethnomedical and ethnopharmacological practices throughout different cultures of the world (Jafari et al. 2016). Satureja species have long been used in folk medicine as a tonic and carminative for treating intestinal ailments, stomach problems, and muscle pain. The antibacterial, antifungal, antioxidant, stimulatory-to-reproduction, and anti-diabetes actions define the medical usage of this plant. Additionally, Satureja plants have a strong inhibitory impact on both Gram-positive and Gram-negative bacteria. (Jafari et al. 2016). Different Satureja species parts, including flowers, roots, stems, leaves, and seeds are used to treat various infectious diseases (Güllüce et al. 2003). Different regions employ roots and aerial parts to alleviate headaches or treat respiratory conditions like asthma and coughing. Plant extracts are used, either alone or in conjunction with other essential oils, in the pharmaceutical, perfume, and cosmetic industries (Momtaz & Abdollahi 2008).

In the recent study conducted by Matejić et al. (2020), the authors attempted to understand the use of herbal drugs in traditional pharmacopoeia to treat some of the most common diseases in Eastern and South-Eastern Serbia regions. Results indicated that *S. montana* is among the species that were identified to be used in ethnomedicine in the selected villages. Among the other identified species, *S. montana* was used to treat respiratory problems: productive cough, bronchitis, and chills.

2.4.6. Medical properties

The essential oils of *S. montana* naturally contain 25 - 415 ppm of methyleugenol. On the use of herbal medicines containing methyleugenol, the European Medicines Agency released a public statement. While this compound is safe for use as flavouring agent in foods and has anticonvulsant, anaesthetic, myorelaxant and hypothermic properties. Methyleugenol is a naturally occurring genotoxic carcinogen with a DNA potency. Therefore the exposure of this compound to sensitive groups should be minimised and further investigation is also required for the toxicological assessment for topical and external use (EMA 2005). Meanwhile research continues to explore and exploit the medicinal potential of *S. montana*.

Satureja species are rich in metabolites such as monoterpenes including thymol, carvacrol, cymene, flavonoids, tannins, acids, and exudates indicate the presence of antimicrobial activities from which pharmacological properties such as antioxidant, anti-inflammatory and analgesic, and anti-hypercholesterolemic activities can be attributed (Kurkcuoglu et al. 2001; Ghannadi 2002; Amanlou et al. 2005; Mchedlishvili et al. 2005).

Diabetes is today's most challenging health problem recognized worldwide. Pavlović et al. (2022) studied the antidiabetic potential of 18 Lamiaceae species including *S. montana*. Results have revealed the presence of 25 secondary metabolites having antidiabetic potential. *S. montana*, was identified as one of the most promising candidates among the species tested.

The presence of Thymoquinone (TQ) secondary metabolite in some plant species including in *S. montana* was recently discovered in the study conducted by Butnariu et al. (2022). TQ is known for its pharmacological properties including anti-inflammatory, antioxidant and anticancer. Specifically, the study focused on the effects of TQ in treating pancreatic cancer. Results revealed promising findings that need further investigations (Butnariu et al. 2022).

2.4.7. Application in plant protection

Satureja spp. can be utilized in plant protection as valuable sources for essential oils rich in thymol and carvacrol. These are valuable botanical pesticides used against insects and pathogens (Liu et al. 2017).

A study conducted by Navarro-Rocha et al. (2020) attempted to develop a chemically stable plant of *S. montana* through pre-domestication to be able to extract essential oils to produce biopesticides. The process helped in increasing the extraction of hydrodistilled oil yield while maintaining a stable yield of dry material. Results showed that oil yield increased in the pre-domesticated plants. Moreover, the pre-domestication process helped in increasing the presence of compounds such as β-myrcene, α- and γ-terpinene, p-cymene, thymol and β-bisabolene, but decreased the presence of α-thujene and carvacrol. Steam distillation however, showed increased levels of α-thujene, α-pinene, α-terpinene, p-cymene and trans-caryophyllene, and decreased borneol, thymoquinone thymol and β-bisabolene. Finally, pressure of steam distillation increased αterpinene, thymol and carvacrol. The study conducted against selected insects/pests revealed that the most active oil compounds were obtained through hydrodistillation.

Satureja montana, essential oil was also recently studied on tomato plants. A study conducted by Verdeguer et al. (2020), tested the capacity of essential oils extracted from *S. montana* to act as both phytopathogenic and natural food preservative. Major compounds found within the extracted essential oils included carvacrol (24.0 %), γ-terpinene (15.9 %) and p-cymene (14.2 %). The essential oils were tested *in vitro* on several organisms including *Alternaria alternata, Botryotinia fuckeliana, Curvularia hawaiiensis, Fusarium equiseti, F. oxysporum lycopersici, Rhizoctonia solani* and *Verticillium dahlia. S. montana* was able to inhibit the growth of most fungi. It also showed excellent results when applied *in vivo* for antifungal tests in Cherry tomatoes and kaki "Persimmon" against *A. alternata.* Sage indicated its capacity to act as an effective post-harvest antifungal agent.

Oliveira-Pinto et al. (2022) investigated the effects of *S. montana* essential oil against *Xanthomonas euvesicatoria*, which is an etiological agent of bacterial spot of tomato. The study was conducted on both molecular and physiological levels. Results indicated that essential oils from *S. montana* include antioxidants which can reduce the levels of superoxide. Some of the most important components include caffeic acid derivatives, including rosemarinic acid and p-coumaric acid; in addition to carvacrol which is an important biologically active phenol monoterpenoid. Also, results indicated that both essential oils and combined treatments had

positive effect on reducing infection evolution in leaves. Results also indicated that when these products are applied to healthy plants, they help in enhancing the overall molecular upregulation of the plants defense pathways, hence increasing their capacity to prevent potential diseases.

2.4.8. Application in the food industry

Antioxidants and antimicrobials extracted from essential oils are increasingly becoming important natural additives in the meat industry as they are known for their capacities to increase shelf life (Alirezalu et al. 2020). Essential oils extracted from aromatic plants including *Stureja montana* have been found to have good antibacterial effect in food preservation. This is mainly due to its good antimicrobial and antioxidant activities in addition to its antibacterial mechanism (Santos et al. 2019). Carvacrol, one of the most abundant compounds present in essential oil extracted, appears to have promising results in the food industry and specifically in the meat industry (Sow et al. 2017; He et al. 2019).

Cui et al. (2021), demonstrated the effectiveness of using antibacterial packaging made from methyl-β-cyclodextrin/*Satureja montana* L. essential oil to examine its effect on preserving meat samples. Results showed promising outcomes as MCD/SEO-SSPS hydrogel produced during the experiment could be considered as a safe and effective food preservative of chilled meat.

Satureja montana byproducts were also tested for their effectiveness against *Escherichia coli*, *Salmonella enterica* sv Anatum, and *Staphylococcus aureus* which are pathogens that impact poultry products. Carvacrol, the most abundant byproduct obtained from *S. montana* essential oils, showed promising results that can even be exploited in the livestock sector (Santos et al. 2019).

Other effects of *S. montana* essential oils and supercritical extracts were tested on pH, lipid oxidation, microbial growth, and sensory quality of fresh pork sausages. The application of essential oils and their supercritical extracts reduced the proliferation of Enterobacteriaceae. The meat treated with the supercritical extracts showed better results than the ones treated with simple essential oils. These results are in line with the previous ones and show promising potential for the use of *S. montana* essential oils as food preservatives (Šojić et al. 2019).

Similar results were obtained when applying different experimental models to determine the antibacterial and anti-listerial effects of *S. montana* essential oils. *In vitro* experiments proved the effects of essential oils against *Listeria monocytogenes* which is a foodborne pathogen and highlighted the interactions between oil mixtures. *In situ* experiments also proved that essential

oils do indeed have an anti-listerial effect on red wine marinated meat (Vasilijević et al. 2019). Essential oils offer several antimicrobial mechanisms of action, and synergism between essential oils decreases the amount needed to kill bacterial pathogens. The volatile character of essential oils is a highly experimented issue as these volatile compounds need to be present in order to be effective.

3. Aims of the thesis

Main aim of the thesis:

The main objective of the research was the induction of polyploidy in *Thymus vulgaris* and *Satureja montana* diploid plants under *in vitro* conditions to obtain new autopolyploid genotypes for improving horticultural and biochemical features of these species.

Specific aims of the thesis:

I. To evaluate the effect of oryzalin on the induction of polyploidy in four concentrations of 20, 40, 60 and 80 μM dissolved in 1 % DMSO for 24 and 48 h treatment time duration under *in vitro* conditions.

II. To determine the ploidy level in the affected plants using Partec PAS flow cytometer (Partec GmbH, Münster, DE) analysis.

III. To evaluate the morphological, biochemical, anatomical and physiological differences between new autopolyploid genotypes and diploid plants.

IV. To determine the genetic stability of new autopolyploid genotypes by periodic repetition of flow cytometric analysis and by repeating biochemical analysis.

4. Hypotheses

I. Oryzalin is an effective antimitotic agent for the induction of polyploidy in *T. vulgaris* L. and *S. montana* L.

II. Flow cytometry analysis determined ploidy level changes in measured autopolyploid genotypes.

III. Autopolyploid genotypes show differences in the morphological, biochemical, anatomical and physiological analyses compared to diploid control plants.

IV. Autopolyploid genotypes are genetically stable after growing in *ex vitro* conditions and maintain improved horticultural and biochemical features.

5. Materials and methods

5.1. Plant material

Plant material was obtained from the collection of medicinal plants from the Botanical Garden (BG) of the Faculty of Tropical AgriSciences (FTA), at the Czech University of Life Sciences Prague (CULS Prague). *Thymus vulgaris* (2n = 2x = 30) is registered under the code 343. and *Satureja montana* (2n = 2x = 30) is registered under the code 334. in periodic publications of the Index Seminum of the BG, FTA, CULS Prague.

The plant material (stems with several nodes) was taken from one mother plant. Before the polyploidization experiment, the plant material was multiplied (cloned) in *in vitro* conditions, see chapter **5.1.2**.

5.1.1. *In vitro* somatic polyploidization

Polyploidization of *T. vulgaris* and *S. montana* was carried out in the Laboratory of Plant Tissue Culture in the department of Crop Sciences and Agroforestry in the Faculty of Tropical AgriSciences in the Czech University of Life Sciences. The polyploidization of *T. vulgaris* experiment took place between 2017 and 2019 and that of *S. montana* was carried out between 2019 and 2021.

5.1.2. *In vitro* culture establishment and propagation

Nodal segments (1.5 cm long) from a single mature plant were sterilized and then cultivated in test tubes on MS medium (Murashige & Skoog 1962) supplemented with 3 % (w/v) of sucrose and 0.8 % (w/v) of agar under *in vitro* conditions. The medium was autoclaved at 121 °C in 100 kPa for 20 min. Nodal segments were washed thoroughly under running distilled water for 1 h, soaked with 70 % ethanol for 30 s, sterilized with 1 % NaClO solution containing two drops of Tween 20 for 25 min and then rinsed 3 times with sterilized distilled water. No plant growth regulators (PGRs) were added to the culture media. The cultures were incubated in a cultivation box (POL-EKO ILW350/350 STD) at 25/20 ± 0.3 °C and a 16/8 h (light/dark) photoperiod with a light intensity of 2500 lx provided by cool white, fluorescent lamps (Philips LT5 14 W/840). After shoot regeneration from meristems, nodal segments of *T. vulgaris* were regularly sub-cultured every 30 days, and nodal segments of *S. montana* were regularly sub-cultured every 40 days, in test tubes on MS medium clear of PGRs.

5.1.3. Polyploidization procedure

Prior to polyploidy induction, 400 nodal segments of *T. vulgaris* and 200 nodal segments of *S. montana* (cca 1.5 cm long), were cultivated for 2 days in 600 ml beakers on 150 ml of MS medium free of plant growth regulators. The nodal segments were subsequently treated for 24 and 48 h with 0, 20, 40, 60 and 80 μM of oryzalin dissolved in 1 % dimethyl sulfoxide (DMSO) solution. A total of 40 nodal segments of *T. vulgaris* were treated in each concentration (0, 20, 40, 60 and 80 μM) for two-time intervals, and a total of 50 nodal segments of *S. montana* were treated in 0 and 20 μM concentration for two-time intervals. After the treatment, influenced nodal segments were removed from the oryzalin solution, rinsed three times in sterilized distilled water and cultivated individually on the same multiplication medium in test tubes for the regeneration of the new shoots. Three months after multiplication, the ploidy level was determined using a flow cytometer. Polyploidization efficiency and survival rates were calculated accordingly:

Efficiency = (Number of polyploid plants / Total number of plants) x 100

Viability = (Number of viable plants / Total number of plants) x 100

5.1.4. Detection of ploidy level

Ploidy levels were determined using flow cytometry analysis for which small parts of leaf tissue were chopped using a razor blade in a Petri dish containing 500 μL of Otto I buffer (0.1 M $C_6H_8O_7$, 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; as such, 1 mL of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_2O$) containing fluorescent dye DAPI 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 3000 nuclei and were recorded using a Partec PAS flow cytometer (Partec GmbH, Münster, DE) equipped with a high-pressure mercury arc lamp. Histograms of DNA content were evaluated using the Flomax software package. The stability of the ploidy level was retested every 6 months over a period of three years, two years of which for *T. vulgaris* plants growing on field conditions.

5.1.5. *In vitro* morphological analysis of *S. montana*

In vitro morphological analysis was performed on *S. montana* plants to measure growth rate difference between the autotetraploid genotypes and the diploid plants during 4 weeks in periods of ± 7 days.

5.1.6. Transfer to *ex vitro* conditions

In vitro cultivated plants with well-developed root systems (diploid and tetraploid) were removed from MS medium, thoroughly rinsed with distilled water to clear medium from roots and transferred to greenhouse with average temperature of 22.5 °C, and relative air humidity range from 70 % to 80 %. Plants were placed in plastic pots (5 × 5 cm) and were maintained for 1 week covered with plastic cups under high humidity and then the humidity was gradually lowered (Fig. 4). The substrate in the pots was created by mixing the following components. Sand 20 %, soil 20 %, white and black peat 20 % (0 - 20 mm), moss:vermiculite 20 %, and compost 20 % (0 - 10 mm) (1:1:1:1;v/v) creating a mixture for an optimal amount of basic nutrients and trace elements. No fertilization was used across the experiment whether for plants growing in the green house or on field conditions.

Figure 4. *Satureja montana* transfer to *ex vitro* conditions

5.1.7. *Ex vitro* quantitative and morphological analyses of *T. vulgaris*

Quantitative (fresh and dry weight) and morphological (plant height, number of branches, main plant thickness, branch thickness, length of branches, internodal distances of main stem, internodal distances of branches, leaf length, leaf breadth and leaf thickness) analyses of *T. vulgaris* were done after 12 months of growth under greenhouse conditions. These comparisons were arranged in a completely randomized design with ten replications for each of the diploid and tetraploid plants.

5.1.8. Transfer of *T. vulgaris* plants from greenhouse to field conditions

Vegetative propagation was performed using approximately 10 cm long cuttings from mother plants for the growth of new plants. First, the ploidy level of two selected mother plants were tested. Then a diploid and an autotetraploid were confirmed by flow cytometry. Fresh green cuttings from these mother plants were propagated into a rectangular wooden box (30 × 60 cm) containing a sand:soil:peat moss:vermiculite (1:1:1:1;v/v) mixture. Rooting was naturally induced by maintaining high soil humidity. Plant plastic labels were placed into the sides of the wooden boxes and used to elevate a transparent vegetable greenhouse foil (Fig. 5). The created dome resembling a mini foil greenhouse, insured high temperature and humidity required for rooting and growth initiation. The whole setup was placed in a regular greenhouse temperature of 22.5 °C, and relative air humidity ranging between 70 % and 80 %. After three weeks, the rooted plants were planted into separate plastic pots (5 × 5 cm) containing a sand:soil:peat moss:vermiculite (1:1:1:1;v/v) mixture (Fig. 5). The potted plants were left for 6 months to grow in greenhouse conditions. In March 2020, diploid and autotetraploid plants were transferred to field conditions.

Figure 5. Vegetative propagation of *T. vulgaris* autotetraploid genotypes and control plants

5.1.9. Field quantitative and morphological analyses of *T. vulgaris*

Quantitative (fresh and dry weight) and morphological (plant height, number of branches, main plant thickness, branch thickness, length of branches, internodal distances of main stem, internodal distances of branches, leaf length, leaf breadth and leaf thickness) analyses of *T. vulgaris* were repeated for diploid and autotetraploid plants, after growing in field conditions. These comparisons were arranged in a completely randomized design with ten replications for each of the diploid and tetraploid plants.

5.2. Biochemical analyses

5.2.1. Initial essential oil isolation and analysis in 2019

Essential oils were extracted from dried samples by hydro-distillation using a Clevenger-type apparatus for three hours. The samples were then dried over an anhydrous sodium sulphate and stored in airtight glass vials in the dark at 4°C up until chemical analysis.

The chemical composition was quantitatively analysed using a gas chromatography Agilent 6890A with a flame ionization detector (FID), while individual components were identified using an Agilent 6890A GC coupled with a quadrupole time-of-flight (Q-TOF) mass detector. Both instruments were equipped with a non-polar HP-5MS (30 m x 250 μM x 0.25 μM) column (Agilent, Santa Clara, CA, USA). One millilitre of sample diluted 1:1000 in hexane was injected in a split ratio of 12:1 into an inlet preheated to 250°C. Helium was used as a carrier gas at the flow rate of 1 mL/min. The temperature program started at 60°C for 3 min, then increased up to 231°C at the rate of 5°C/min and kept constant for another 10 min. The FID detector was heated to 250°C. MS analysis was carried out in full scan mode, and the electron ionization energy was set to 70 eV. The identification of individual components was based on the comparison of their mass spectra with the relative retention indices, the National Institute of Standards and Technology Library (NIST, USA) and literature (Adams 2007) as well as by co-injection of authentic standards.

5.2.2. Second essential oil isolation and analysis

Dried samples of thyme aerial parts were submitted to hydro-distillation with a Clevenger-type apparatus for three hours. The distillation was performed three times for both diploids and tetraploids using three separate sample mixtures each consisting of individual plants. The collected essential oils were dried over anhydrous sodium sulphate and stored in airtight glass vials at 4 °C until further analysis.

The chemical composition of essential oils was determined using an Agilent 7890A gas chromatograph (GC) coupled with an Agilent 5975C single-quadrupole mass detector and a relative quantification was based on the measurements on an Agilent 7890A GC with flame ionization detector (FID). Both GCs were equipped with a non-polar HP-5MS (30m x 250 μm x 0.25 μm) capillary column (Agilent, Santa Clara, CA, USA). Samples were diluted 1:1000 in hexane and injected into an inlet heated to 250°C. The split ratio was 12:1 with 1 μL sample volume. The temperature program started at 60°C for 3 min, then increased 231°C at the rate of 3°C/min and then kept constant for 10 min. Helium was used as a carrier gas with the flow rate of 1 mL/min. The FID detector temperature was 300°C. The MS analysis was performed in full-scan mode with the electron ionization energy set at 70 eV.

The identification of essential oil constituents was based on a comparison of their mass spectra and Kovats retention indices with the National Institute of Standards and Technology Library (NIST, USA) and literature (Adams 2007). The relative percentage content of the EO components was calculated by dividing the individual peak area by the total area of all peaks. The identification of 15 components (see Tab. 8) was further confirmed by co-injection of authentic standards obtained from Sigma–Aldrich (Prague, Czech Republic).

5.2.3. Antioxidant activity analyses

5.2.3.1. DPPH radical-inhibiting assay

The DPPH radical scavenging assay was performed using a slightly modified method previously described by (Sharma and Bhat 2009). A two-fold serial dilution of each sample was prepared in methanol in 96-well microtiter plate. To start the radical-antioxidant reaction, an amount of 100 μL of freshly prepared 0.25 mM diphenyl-2-picryl-hydrazyl (DPPH) solution dissolved in methanol (MeOH) was added to each well creating the following concentration range of each sample: 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, and 0.5 μg/mL. The plate was incubated in the dark at laboratory temperature for 30 min. Absorbance was measured at 517 nm using Synergy H1 multiplate reader (BioTek, Santa Clara, USA). Trolox (at same concentrations as samples) was used as a positive control, whereas MeOH as a negative control. Results were expressed as half-minimal inhibitory concentrations (IC50 in μg/mL) and were afterwards recalculated to Trolox equivalents (μg TE/mg DW).

5.2.3.2. Oxygen radical absorbance capacity (ORAC)

The ability of samples to retard the AAPH-induced decay of FL was established by slightly modifying the method developed by Ou et al. (2001). Initially, black absorbance 96-well microtiter plates were filled with 200 μL of distilled water, in order to provide better thermal mass stability of the measured wells, as previously suggested by Held (2005). All reagents and samples were prepared in 75 mM phosphate buffer (pH 7.0). Each sample in an amount of 25 μL was dissolved in 150 μL FL (48 nM) and incubated in the dark for 10 min at 37°C. FL decay was initiated by the addition of 25 μL AAPH (153 mM). Extracts were tested at a concentration of 3.125 μg/mL. Standard calibration curves of Trolox were obtained at five concentration levels (0.5, 1, 2, 4, and 8 μg/mL; final concentration). Fluorescence changes were measured in 1-min intervals for 120 min with emission and absorbance wavelengths set at 485 nm and 520 nm, respectively. Quantification of the antioxidant capacity was calculated as the area under the calibration curve, as previously proposed by Cao and Prior (Cao & Prior 1998) and expressed as Trolox equivalents (μg TE/mg DW).

5.2.3.3. Trolox equivalent antioxidant capacity (TEAC)

The TEAC was determined by slightly modifying the improved ABTS radical cation decolourization method described by Re et al. (1999). The ABTS radical was generated by mixing 5 μL ABTS (7 mM) with 500 μL APS (245 mM) and incubating the solution overnight in the dark at room temperature. Before the experiment, the ABTS radical was diluted (to approx. 1 % v/v) in PBS buffer until it reached absorbance reads ≈ 0.700 at 734 nm. An amount of 10 μL of each sample was transferred to the 96-well microtiter plates. Afterwards, 190 μL of the ABTS radical was added to each well. Plates were incubated in the dark at room temperature for 5 min. Absorbance was read at 734 nm. Extracts were tested at a concentration of 2.5 μg/mL. A calibration curve of Trolox was acquired using seven concentrations (0.156, 0.313, 0.625, 1.25, 2.5, 5, and 10 μg/mL; final concentration). Results were expressed as Trolox equivalents (μg TE/mg DW).

5.2.3.4. Total phenolic content

Total phenolic content was established by slightly modifying Folin–Ciocalteu reagent-based method previously developed by Singleton et al. (1998). Each sample in a volume of 100 μL was transferred to 96-well microtiter plate. Subsequently, 25 μL of undissolved Folin-Ciocalteu reagent was added to each well and the plates were placed on an orbital shaker for 10 min. The reaction was started with the addition of 75 μL of 12 % (w/v) Na_2CO_3. Plates were incubated

in the dark at 37°C for 120 min. Absorbance was measured at 700 nm. Extracts were tested at a concentration of 50 µg/mL. Gallic acid was used as a standard; seven concentrations (1, 2, 4, 8, 16, 32, and 64 µg/mL; final concentration) were used to create the calibration curve. Results were expressed as gallic acid equivalents (µg GAE/mg DW).

5.2.3.5. Total flavonoid content

Total flavonoid content was determined using a modified aluminium chloride ($AlCl_3$) method developed by Christ and Müller (1960). An amount of 100 µL of each sample was mixed with 100 µL of 10 % (w/v) $AlCl_3$ in a 96-well microliter plate. Plates were incubated in the dark at room temperature for 60 min. Absorbance was measured at 420 nm. Extracts were tested at a concentration of 1.250 µg/mL. Quercetin was used as a standard; the calibration curve was created from seven concentration levels (1.563, 3.125, 6.25, 12.5, 25, 50, and 100 µg/mL; final concentration). Results were expressed as quercetin equivalents (µg QE/mg DW).

5.3. Anatomical analyses

5.3.1. Quantitative wood anatomy

Anatomical features of diploid and autotetraploid thyme were analysed based on high-resolution digital images of anatomical cross sections prepared following Gärtner and Schweingruber (2013). Stem cuts of diploid and polyploid were cut just above the root collar (1 cm above the soil) to avoid wood wedging and branching. Samples were chemically treated in alcohol (ethanol 96 % and 100 %) and xylol, followed by paraffin embedding that ensures stability and preservation of cell structure during sectioning. Embedded stem cuts were then transversally cut with a rotary microtome (Leica RM2245, Heidelberg, Germany) using Feather N35 blades (Feather Safety Razor Co., Ltd, Osaka, Japan) at 12 µm. Finally, microsections were stained with safranin (1 % in distilled water) to increase contrast and were permanently mounted on glass slides using Eukitt.

Images of the microslides were captured with a NIKON camera at 10x magnification (Nikon DS-Fi1c-L3, Shanghai, China) mounted on a motorized microscope (Ni-Ci Eclipse, Nikon Corp., Tokyo, Japan) at a resolution of 2.94 pixels/µm. Main anatomical features (i.e. mean cell number, lumen size, and cell-wall thickness) were obtained using the integrated measurement features of the NIS-AR Image Analysis software (Nikon), based on region of interest (i.e. ROI) and binary thresholding.

5.3.2. Stomata density

The ‘Nail varnish method’ was used to measure the stomata density on the abaxial epidermal side (Kolodziejek & Michlewscka 2015; van den Top et al. 2018). Fresh thyme leaves were selected from new shoots of diploid and tetraploid plants. Samples were taken from the middle, widest part of the leaves, with five repetitions performed per plant. After applying transparent nail varnish to the abaxial side of each leaf, the leaves were left 20 minutes to dry. Transparent tape was gently used to peel off the dried layer of nail varnish stomata impression. Then each tape was placed onto a microscope slide. Stomata on surface view of the lower epidermis was observed using a CKX41 Inverted Microscope (Olympus Corp. Tokyo, Japan), 40x magnification, and photographed using (Olympus C7070WZ) camera. Stomata density was counted using 25x square grid.

5.3.3. Glandular trichome anatomy

Fresh leaves at various stages of maturity were selected for observation from field grown plants. The distribution and external morphology of the glandular trichomes were examined according to Huang et al. (2008). These examinations were done using a Wild Heerbrugg Macroscope M420 1,25x fitted with a Cannon DS126621 Digital SLR 18.0MP.

5.4. Physiological analysis

5.4.1. Chlorophyll estimation

Chlorophyll contents of diploid and tetraploid plants were estimated using the protocol by Mosa et al. (2018) with slight modifications. Fresh leaf samples were crushed into powder in liquid nitrogen. Then, 5 ml of 80 % acetone was added to 300 mg of grounded powder and placed in dark on a shaker for 15 mins, followed by centrifugation at 3000 RPM for 15 mins at 4°C. Afterwards, supernatant was collected in a fresh centrifuge tube and diluted with 80 % acetone in a 1:5 ratio. Absorbance was measured using a Spectrophotometer at two wavelengths: 663 nm for chlorophyll a and 645 nm for chlorophyll b; the values were then utilized to calculate the chlorophyll contents.

5.5. Statistical analyses

Statistical analysis of the data obtained from quantitative and morphological evaluation of the tetraploid and diploid (control) plants was performed using the statistical software InfoStat 2016 (National University of Cordoba, Argentina). The one-way ANOVA (analysis of variance) test was used to compare individual groups. The Tukey post-hoc test was applied to

find means that were significantly different for each variant. Differences were considered significant at $P<0.05$. Results are expressed as mean ± SD (standard deviation) of three parallel measurements where each sample was tested in triplicate. Anatomical differences were statistically quantified using the R core team software (R Development Core Team 2021). The mean differences between diploid and tetraploid plants were compared using the Mann–Whitney test for each variable at $p < 0.05$. Standard deviation (±SD) and coefficient of variation (CV) were estimated. Physiological measurements were analysed using the program Statistika 12. The one-way ANOVA (analysis of variance) test was used to compare individual groups. Other statistical analyses were done using Excel statistics.

6. Results

6.1. *Thymus vulgaris* L.

6.1.1. Polyploidization efficiency

The survival rate of 320 oryzalin-treated plants in total ranged from 5 % to 32.5 %, whereas the highest rate was obtained at the lowest oryzalin concentration 20 μM (Tab. 2). The thyme regeneration percentage was also affected by the exposure duration, with a higher percentage in the 24 h group than in the 48 h group (Tab. 2). Of the 320 plants treated with oryzalin, 60 (18.75 %) plants survived.

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

Oryzalin concentration (μM)	No. of explants treated (nodal segments)	Treatment duration (h)	Survival rate (%)	No. of tetraploid plants	Polyploidization efficiency (%)
0	40	24	100	0	0
	40	48	100	0	0
20	40	24	32.5	0	0
	40	48	25	0	0
40	40	24	25	0	0
	40	48	25	0	0
60	40	24	12.5	0	0
	40	48	5	0	0
80	40	24	32.5	0	0
	40	48	15	3	7.5
Total	320			3	

6.1.2. Flow cytometry analysis

Following three cultivations, the 60 surviving plants were screened by flow cytometry to determine their ploidy levels. Measurements were carried out three months after the oryzalin treatment. Diploid control plants were measured first (Fig. 6). According to the flow cytometric analysis, concentrations of 20, 40 and 60 μM of oryzalin had no effect on polyploidy induction.

Three genotypes (Tv1, Tv2 and Tv3) were obtained at the highest concentration of 80 µM for 48 h (Fig. 7)

With only 3 tetraploid plants obtained out of 40 treated nodal segments in the 80 µM/48 h variant, the polyploidization efficiency was 7.5 %. During propagation, two genotypes (Tv2 and Tv3) did not produce a root system and eventually developed necrosis and perished. Plants with well-developed root systems were further multiplied. In total, 20 tetraploid and 20 control diploid plants were transferred to *ex vitro* conditions. The conversion survival rate of plants in *ex vitro* conditions was 90% and 85 % in control diploid and tetraploid plants, respectively.

Flow cytometric analysis repeated after two years of growing in field conditions to determine the genetic stability of the autopolyploid genotype and its diploid counterpart are shown in Figures 6 and 7.

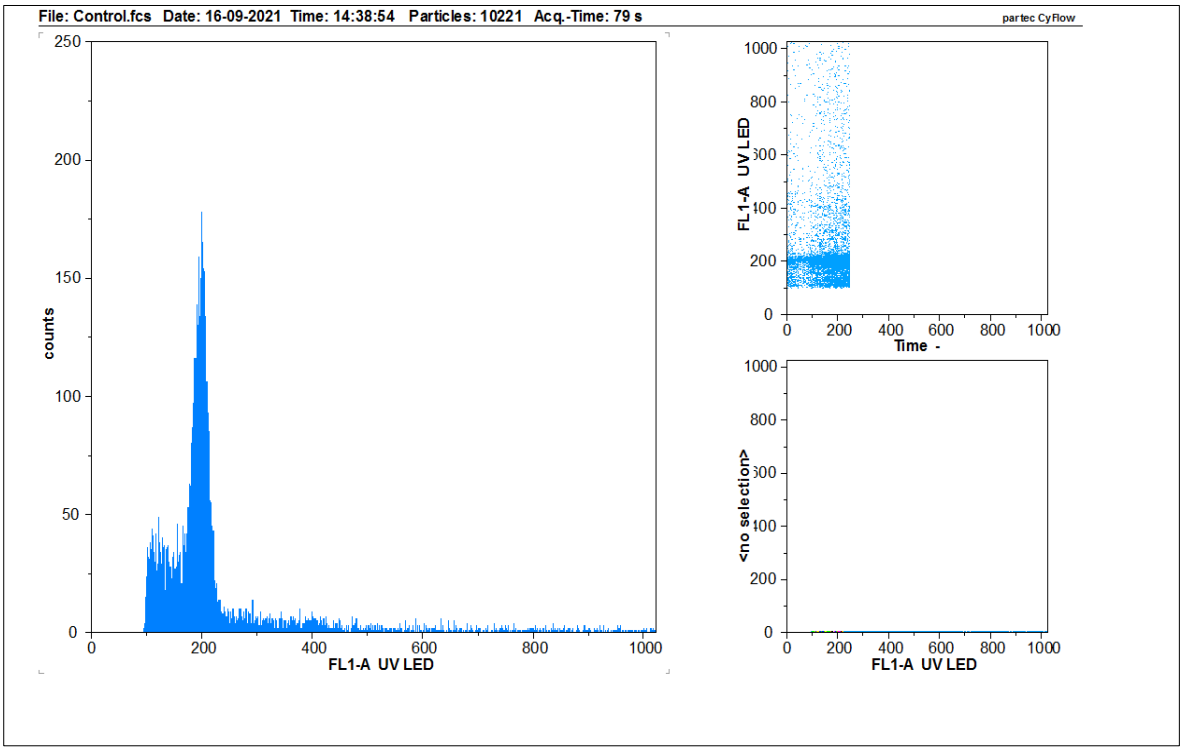

Figure 6. Histogram of relative DNA content with a peak corresponding to G0/G1 nuclei of the control plant on Channel 200

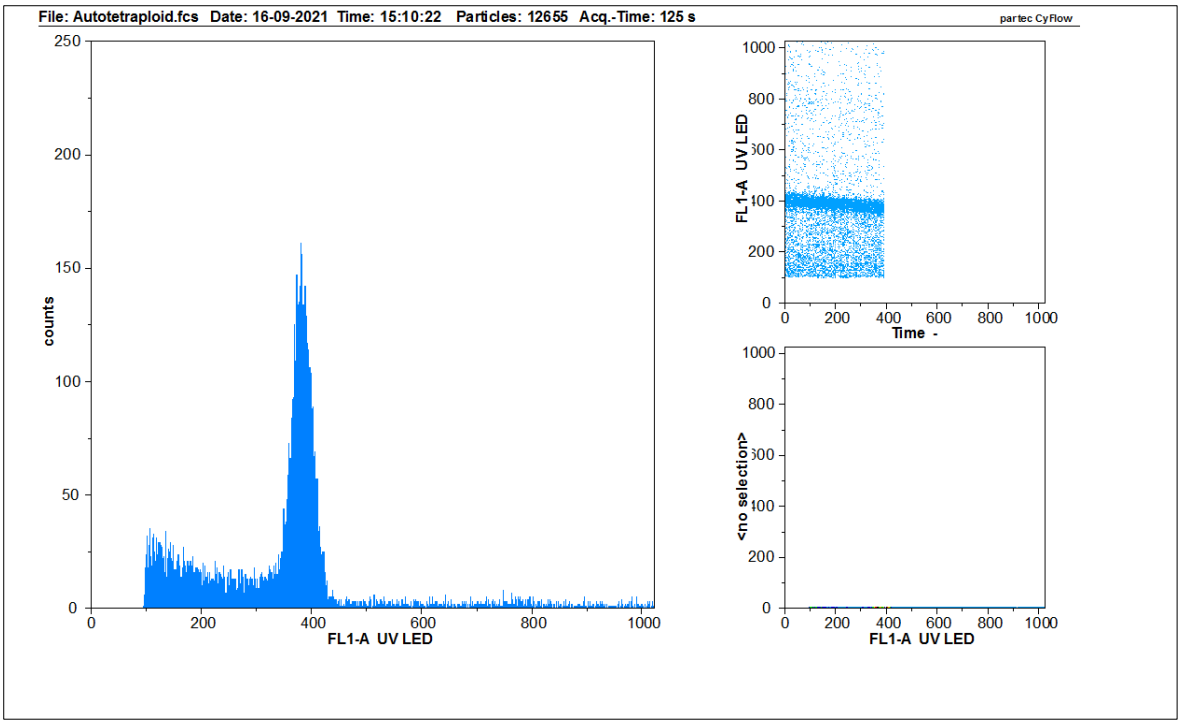

Figure 7. Histogram of relative DNA content with a peak corresponding to G0/G1 nuclei of tetraploid plant on Channel 400

Samples from the control plants and from the autotetraploid plants, growing on field conditions, were collected for analysis. Relative DNA content of diploid *T. vulgaris* (2n = 2x = 30) was set to channel 200 and constant parameters were maintained to follow measurements of influenced plants. Results from repeated measurements confirm the ploidy level of the autotetraploid *T. vulgaris* (2n = 4x = 60) genotype (Fig. 7).

6.1.3. Quantitative and morphological analyses

Newly acquired tetraploids exerted distinctly different morphological characteristic from the mother diploid plants in *in vitro*, green house and field conditions as shown in Figures 8, 9 and 10, respectively.

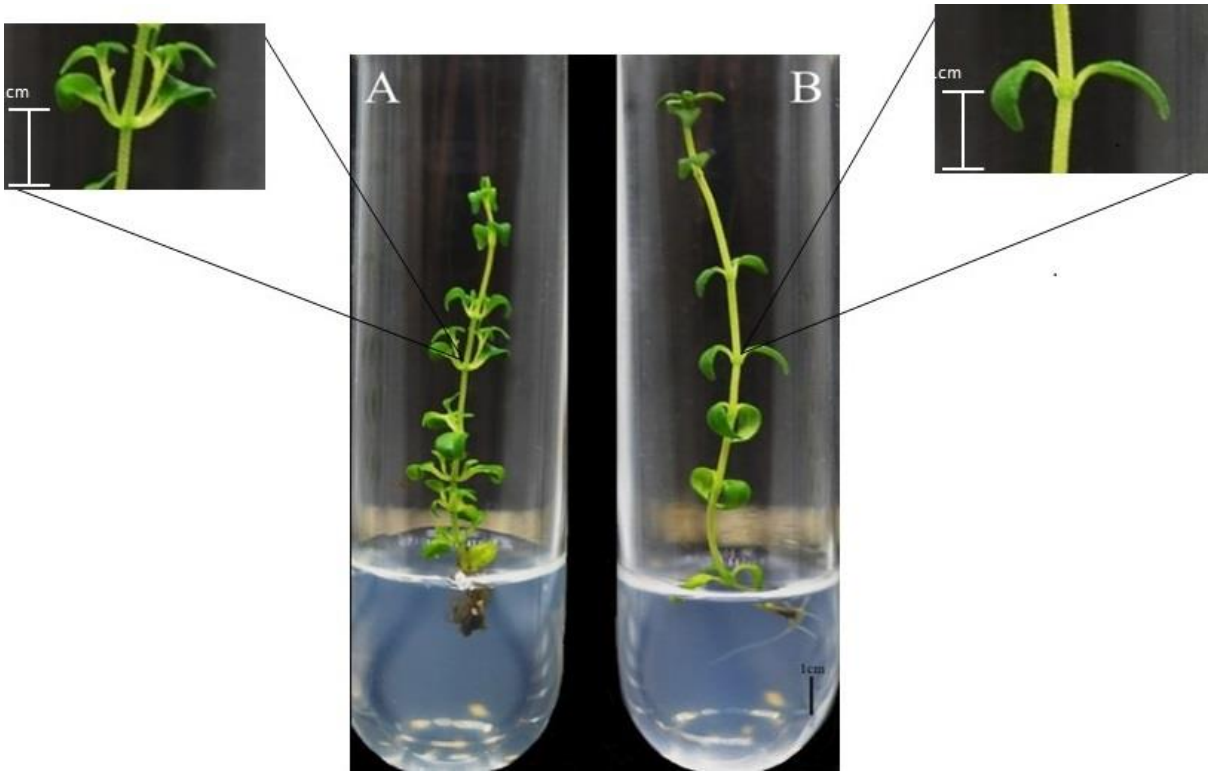

Figure 8. *In vitro* morphological variation between *T. vulgaris* control plant (A) and oryzalin induced tetraploid plant (B) growing in test tubes on MS medium

Figure 9. Morphological variation between *T. vulgaris* diploid plant (A) and oryzalin induced tetraploid plant (B) cultivated under greenhouse conditions

After 12 months of growing under greenhouse conditions, tetraploid plants showed a statistically significant increase in main plant thickness (217 %), leaf length (136 %), leaf breadth (159 %), leaf thickness (145 %) and plant height (200 %) (Tab. 3).

Repeated morphological analysis performed on diploid and autotetraploid *T. vulgaris* plants growing in field conditions showed similar statistically significant differences to the morphological analysis performed in year 2019 for the following parameters: Main plant thickness, leaf (length, width, and thickness), plant height, branch length, and internodal distance of main stem (Tab. 5).

Table 3. Morphological characteristics of diploid and tetraploid plants growing in green house conditions in 2019

Variants	Main plant thickness (mm)	Branch thickness (mm)	Leaf length (mm)	Leaf width (mm)	Leaf thickness (mm)	Plant height (cm)	Branch length (cm)	Internodal distance of main stem (cm)	Internodal distance of branches (cm)
Diploid	1.00±0.20[a]	0.64±0.09[a]	6.25±0.22[a]	2.62±0.18[a]	0.30±0.01[a]	26.23±3.36[a]	12.11±2.89[a]	1.24±0.13[a]	1.03±0.11[a]
Tetraploid	1.57±0.14[b]	0.83±0.06[a]	6.90±0.16[b]	3.61±0.13[b]	0.45±0.019[b]	38.91±3.36[b]	14.41±2.04[a]	1.58±0.09[a]	1.17±0.08[a]

[a,b,c] Means within the same row with different superscripts differ (P < 0.05 – P < 0.001)

These morphological changes in tetraploid *T. vulgaris* led to vigorous growth of the plant, which was translated as an increase in fresh plant weight (257 %) and dry plant weight (300 %) (Tab. 4).

Table 4. Comparison of fresh and dry weight (g) between diploid and tetraploid plants in 2019

Variants	Fresh plant weight (g)	Dried plant weight (g)
Diploid	3.22±1.92[a]	1.00±0.55[a]
Tetraploid	9.72±1.35[b]	2.72±0.39[b]

[a,b,c] Means within the same row with different superscripts differ (P < 0.05 – P < 0.001).

Table 5. Second morphological characteristics of diploid and tetraploid plants growing on the field

Variants	Main plant thickness (mm)	Branch thickness (mm)	Leaf length (mm)	Leaf width (mm)	Leaf thickness (mm)	Plant height (cm)	Branch length (cm)	Internodal distance of main stem (cm)	Internodal distance of branches (cm)
Diploid	1.53±0.03[a]	0.66±0.02[a]	5.94±0.02[a]	2.0±0.06[a]	0.34±0.01[a]	27.44±0.27[a]	7.66±0.02[a]	1.03±0.016[a]	0.55±0.01[a]
Tetraploid	3.14±0.02[b]	1.17±0.03[b]	6.33±0.01[b]	3.03±0.02[b]	0.42±0.01[b]	46.34±0.18[b]	10.93±0.02[a]	1.67±0.016[b]	1.03±0.02[b]

[a,b,c] Means within the same row with different superscripts differ (P < 0.05 – P < 0.001).

Table 6. Second comparison of fresh and dry weight (g) between diploid and tetraploid plants

Variants	Fresh plant weight (g)	Dried plant weight (g)
Diploid	3.99±0.06[a]	1.18±0.02[a]
Tetraploid	9.64±0.15 [b]	2.85±0.01[b]

[a,b,c] Means within the same row with different superscripts differ (P < 0.05 – P < 0.001).

Quantitative analysis of fresh and dry plant weight for the second analysis showed statistically significant differences between diploid and autotetraploid *T. vulgaris* plants growing in field conditions (Tab. 6). These results were similar to those from the year 2019 as there were statistically significant differences in fresh and dry plant weight between diploid and tetraploid in both years (Tab. 4) and (Tab. 6).

Figure 10. Morphological variation between *T. vulgaris* diploid plant (A) and oryzalin induced tetraploid plant (B) growing in field conditions

Three morphological parameters have changed between the first and the second analyses. Branch thickness, internodal distance of branches, and internodal distance of main stem showed statistically significant differences between diploid and autotetraploid plants in the second analysis (Tab. 3) and (Tab. 5).

6.1.4. Biochemical analyses

6.1.4.1. Initial essential oil yield and analysis in 2019

The essential oil content was significantly higher in tetraploid plants. The yields were 0.81 % and 1.19 % w/v for the diploid and tetraploid *T. vulgaris*, respectively.

In our study, the chemical analysis of thyme essential oil in 2019 revealed thymol (30.31 % and 48.32 %), p-cymene (23.33 % and 13.2 %) and γ-terpinene (19.08 % and 12.82 %) as the major components, together representing 72.72 % and 74.34 % of the total oil composition of diploid and tetraploid *T. vulgaris*, respectively (Tab. 7).

Table 7. Chemical composition of essential oils obtained from aerial parts of diploid and tetraploid *T. vulgaris* in 2019

Compound	RI[a]		Tetraploid *(%)*	Diploid *(%)*	Identification[d]
	observed	*published*[b]			
α-Thujene	931	931	1.28 ± 0.001	0.99 ± 0.001	MS, RI
α-Pinene	938	939	1.20 ± 0.002	0.73 ± 0.001	MS, RI, Std
Camphene	953	953	1.35 ± 0.002	0.61 ± 0.001	MS, RI
β-Pinene	980	980	1.16 ± 0.001	1.04 ± 0.003	MS, RI, Std
β-Myrcene	992	991	1.58 ± 0.000	1.62 ± 0.001	MS, RI, Std
α-Terpinene	1019	1018	1.67 ± 0.001	1.64 ± 0.002	MS, RI, Std
***p*-Cymene**	**1028**	**1026**	**13.20** ± 0.006	**23.33** ± 0.011	MS, RI, Std
Eucalyptol	1035	1033	1.69 ± 0.000	2.10 ± 0.001	MS, RI, Std
γ-Terpinene	**1063**	**1062**	**12.82** ± 0.004	**19.08** ± 0.009	MS, RI, Std
Cis-sabinene hydrate	1070	1068	1.38 ± 0.002	1.27 ± 0.001	MS, RI
Linalool	1100	1098	1.77 ± 0.009	2.93 ± 0.008	MS, RI, Std
Camphor	1148	1143	1.54 ± 0.002	0.08 ± 0.001	MS, RI
Borneol	1169	1165	1.75 ± 0.013	1.59 ± 0.004	MS, RI
Thymol	**1294**	**1290**	**48.32** ± 0.046	**30.31** ± 0.009	MS, RI, Std
Carvacrol	**1302**	**1298**	**2.67** ± 0.004	**2.18** ± 0.002	MS, RI, Std
β-Caryophyllene	1422	1418	1.08 ± 0.002	3.58 ± 0.001	MS, RI, Std
Total identified			94.48	93,07	

[a] Kovat's retention indices (HP-5MS capillary column); [b] Data taken from Adams (2007); [c] Mean ± SD (*n*=3); [d] Identification method: MS=based on comparison of mass spectra with those of National Institute of Standards and Technology Library, RI=comparison of retention indices with literature, Std=confirmed by co-injection of authentic standards

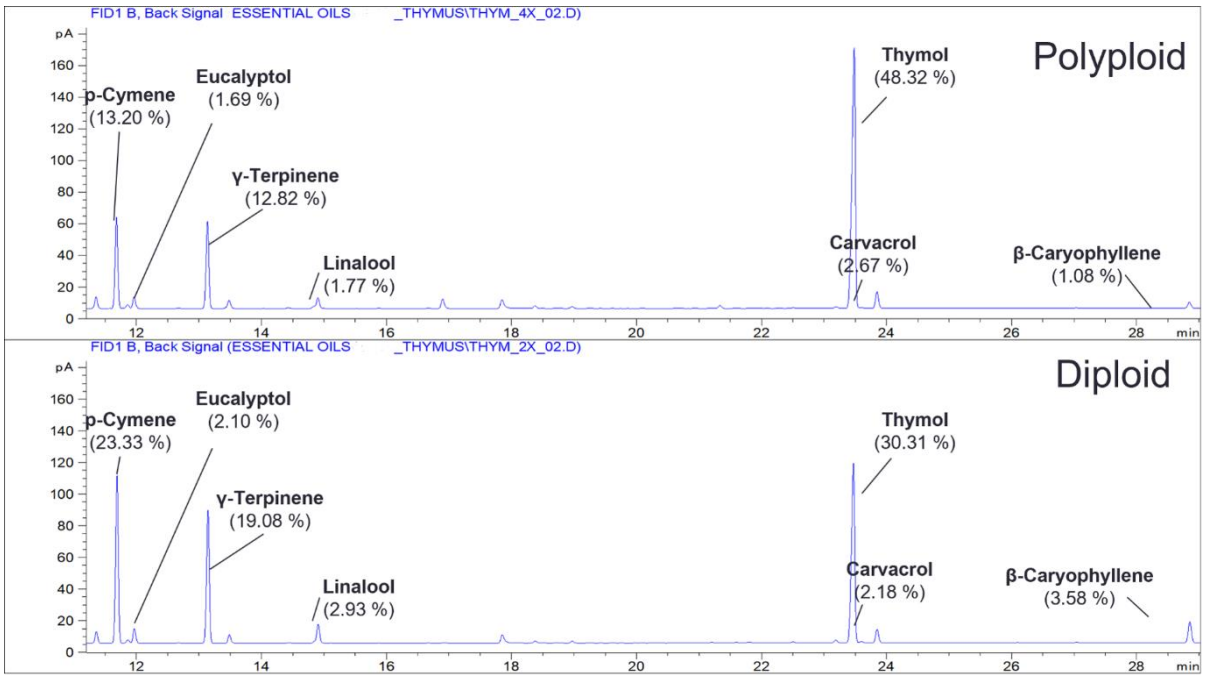

Figure 11. Essential oil composition of diploid and tetraploid *T. vulgaris*

Compared with the diploid plants, there was a remarkable 1.6-fold increase in thymol production observed in the tetraploid plants, followed by camphor production that increased from almost trace content to 1.54 %. This increase was compensated proportionally by the decreased content of thymol precursors p-cymene (10.13 %) and γ-terpinene (6.26 %), followed by β-caryophyllene (2.5 %) and linalool (1.16 %). The changes in the content of other components did not exceed 1 %. When comparing the ratio of the percentage content of the three major constituents, a significant increase in the thymol/p-cymene (from 1.3 to 3.7) and thymol/γ-terpinene ratios (from 1.6 to 3.8) can be noticed in the tetraploid plant (Fig. 11).

6.1.4.2. Second essential oil yield and analysis

The essential oil yields were 1.28 ± 0.03 % for tetraploids and 0.81 ± 0.04 % for diploid plants. In total, 19 and 18 compounds were identified in the essential oils extracted from tetraploid and diploid plants, which constitutes 97.89 and 97.80 % of the total constituents, respectively. Thymol (29.33 % and 50.12 %), p-cymene (32.11 % and 15.33 %), γ-terpinene (13.05 % and 9.56 %), and carvacrol (4.10 % and 5.08 %) were the most abundant components in the

essential oil of diploid and tetraploid plants respectively. Complete compositions are listed in (Tab. 8).

Table 8. Chemical composition of *T. vulgaris* essential oils distilled from aerial parts of diploid and tetraploid plants

Compound	RI[a]		Tetraploid	Diploid	Identification[d]
	observed	*published*[b]	*(%)*[c]	*(%)*	
α-Thujene	930	931	0.89 ± 0.024	1.19 ± 0.050	MS, RI
α-Pinene	937	939	1.13 ± 0.058	1.00 ± 0.088	MS, RI, Std
Camphene	952	953	1.19 ± 0.077	0.65 ± 0.071	MS, RI, Std
β-Pinene	979	980	0.91 ± 0.036	0.87 ± 0.044	MS, RI, Std
β-Myrcene	992	991	1.69 ± 0.066	1.84 ± 0.083	MS, RI, Std
α-Terpinene	1018	1018	1.39 ± 0.053	1.31 ± 0.124	MS, RI, Std
***p*-Cymene**	1027	1026	**15.33 ± 0.874**	**32.11 ± 1.313**	MS, RI, Std
Limonene	1031	1031	0.59 ± 0.019	0.55 ± 0.098	MS, RI
Eucalyptol	1033	1033	2.97 ± 0.120	3.63 ± 0.064	MS, RI, Std
γ-Terpinene	1062	1062	**9.56 ± 0.562**	**13.05 ± 1.745**	MS, RI, Std
Cis-sabinene hydrate	1069	1068	1.12 ± 0.068	0.95 ± 0.033	MS, RI
Linalool	1099	1098	2.02 ± 0.051	3.59 ± 0.128	MS, RI, Std
Camphor	1145	1143	1.77 ± 0.088	n.d.	MS, RI
Borneol	1168	1165	0.21 ± 0.053	0.40 ± 0.017	MS, RI, Std
Terpinen-4-ol	1179	1177	0.49 ± 0.013	0.49 ± 0.024	MS, RI, Std
Thymol	1293	1290	**50.12 ± 0.775**	**29.33 ± 1.674**	MS, RI, Std
Carvacrol	1301	1298	**5.08 ± 0.209**	**4.10 ± 0.312**	MS, RI, Std
β-Caryophyllene	1420	1418	1.15 ± 0.129	2.03 ± 0.703	MS, RI, Std
Caryophyllene oxide	1583	1581	0.29 ± 0.028	0.71 ± 0.125	MS, RI, Std
Total identified			97.89	97.80	

[a] Kovat's retention indices (HP-5MS capillary column); [b] Data were taken from Adams 2007); [c] Mean ± SD (individual measurements of three separately distilled samples); [d] Identification method: MS=comparison of mass spectra with those of National Institute of Standards and Technology Library. RI=comparison of retention indices with literature. Std=identification confirmed by co-injection of authentic standards; n.d.=not detected

6.1.4.3. Antioxidant activity and total phenol and flavonoid content

The yields of extraction for antioxidant activity were 9.58 ± 1.41 % for diploid and 12.41 ± 0.78 % for tetraploid plants (Tab. 9). Antioxidant activity was higher in tetraploid plants compared to diploid plants. The radical scavenging assay (DPPH) used to evaluate the antioxidant activity was 28.77 ± 3.87 % in diploid plants and 43.40 ± 2.41 % in tetraploid plants. The ORAC oxygen radical absorbance capacity, used as a reference for antioxidant effectiveness, was 101.92 ± 8.63 % for diploid plants and 158.03 ± 14.31 % for tetraploid plants. The trolox equivalent antioxidant capacity (TEAC) used for total antioxidant capacity determination was 51.76 ± 9.04 % for diploid plants and 85.16 ± 12.51 % for tetraploid plants (Tab. 9).

Total phenolic and total flavonoid contents were significantly higher in tetraploid plants compared to diploid plants. The total phenolic content (TPC) was 22.05 ± 3.93 % for diploid plants and 37.78 ± 7.93 % for tetraploid plants. The total flavonoid content (TFC) was 1.63 ± 0.10 % for diploid plants and 2.4 ± 0.13 % for tetraploid plants (Tab. 9).

Table 9. Antioxidant activity, and total phenolic and flavonoid content in tested samples (expressed as mean ± SD)

Sample	DPPH	ORAC	TEAC	TPC	TFC
		ug TE/mg DW		ug GAE/mg DW	ug QE/mg DW
diploids					
D1	29.19 ± 6.48	89.67 ± 5.25	47.07 ± 7.48	20.02 ± 3.20	1.46 ± 0.05
D2	33.12 ± 3.05	125.76 ± 13.18	60.69 ± 12.20	25.98 ± 5.10	1.92 ± 0.18
D3	24.02 ± 2.08	90.33 ± 7.48	47.52 ± 7.46	20.17 ± 3.51	1.53 ± 0.08
tetraploids					
T1	45.86 ± 0.37	182.56 ± 19.66	93.25 ± 16.18	40.75 ± 8.68	2.92 ± 0.20
T2	41.82 ± 3.68	148.02 ± 10.82	84.66 ± 12.11	38.07 ± 7.76	2.24 ± 0.11
T3	42.54 ± 3.19	143.53 ± 12.45	77.58 ± 9.26	34.54 ± 7.36	2.04 ± 0.08

6.1.5. Anatomical variation between diploid and tetraploid *T. vulgaris*

6.1.5.1. Qualitative wood anatomy

We found significant differences in anatomical features between the diploid and the tetraploid plants (Fig. 12). The tetraploid generally had a higher number of tracheid cells and larger cell lumen than the diploid, but thinner radial cell walls (Fig. 12b., $p < 0.001$).

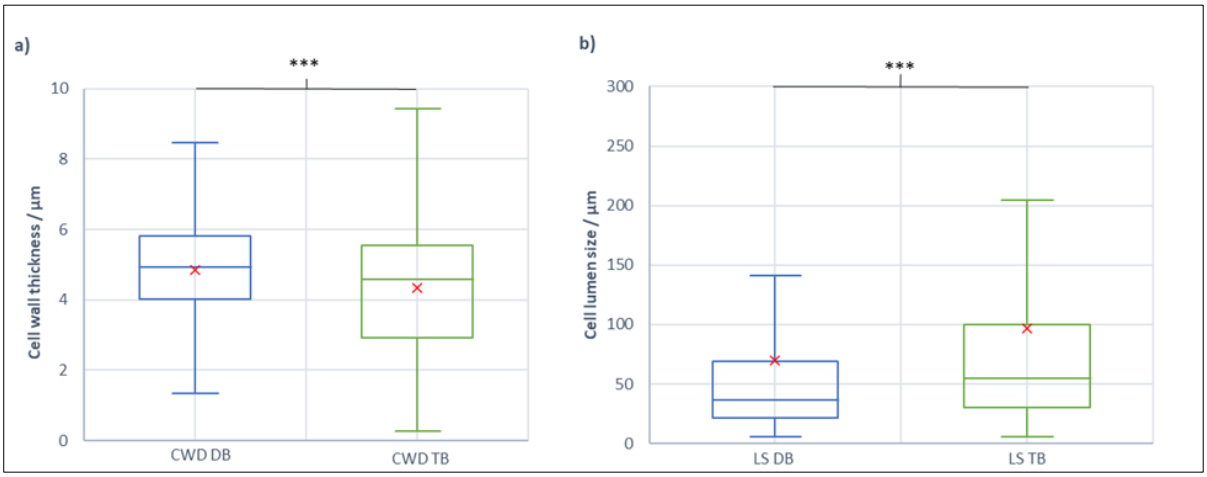

Figure 12. Box-whisker plots of differences in main anatomical features

Statistical differences in (a) cell-wall thickness (i.e., CWD) and (b) cell lumen size (i.e. LS) between diploid and tetraploid plants (i.e. DB for diploid, TB for tetraploid) were tested using Mann-Whitney test at $p < 0.05$. Black asterisks indicate significant differences in mean values.

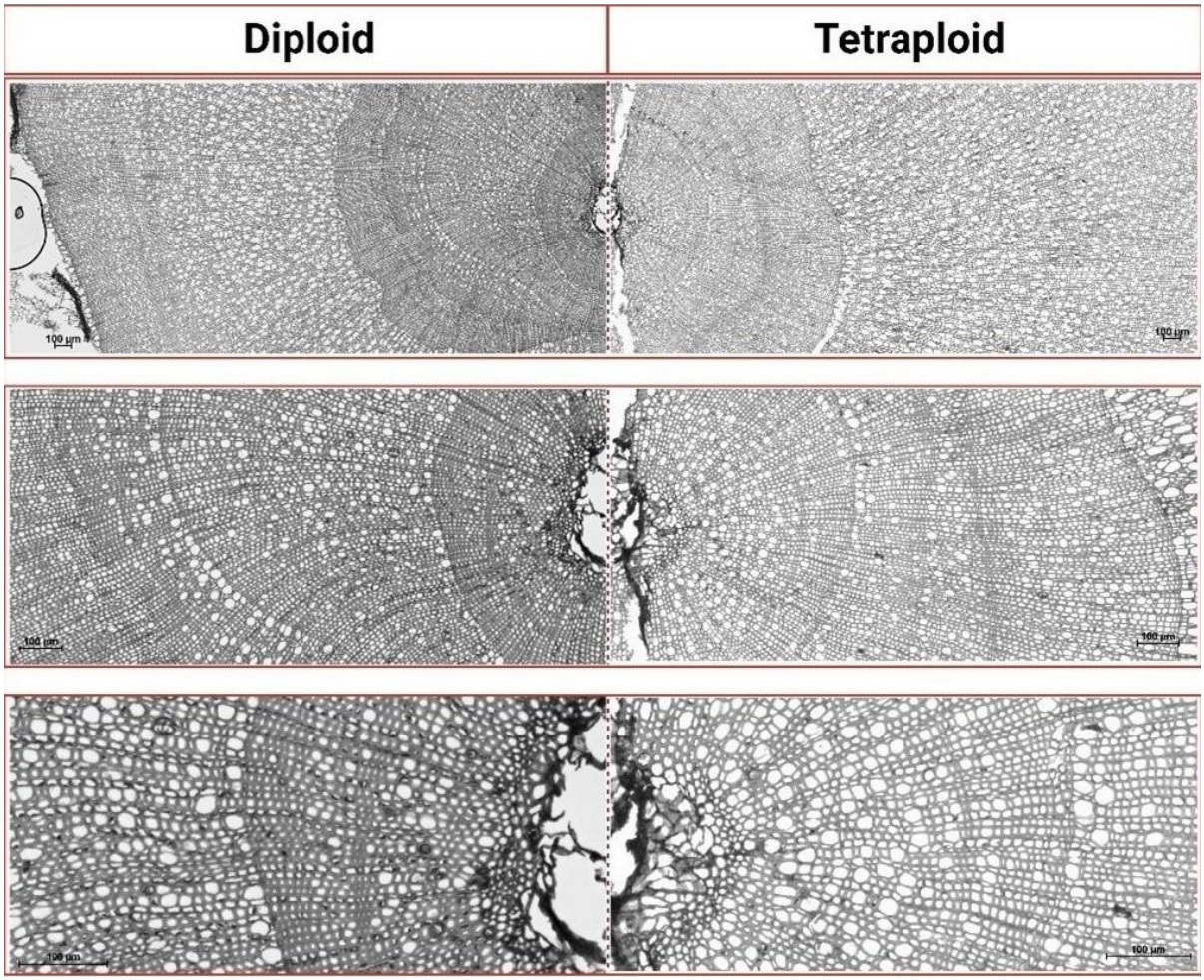

Figure 13. Anatomical cross section of diploid and autotetraploid *T. vulgaris* stem cut

Anatomical cross section in the lateral stem base cut showed the diploid plants to have thicker radial cell walls. But there were fewer tracheid cells with smaller cell lumen in the diploid plants than in the tetraploid plants (Fig. 12a.).

6.1.5.2. Stomata density

Stomata numbers were counted using square grid over microscope images (Tab. 10). Stomata density on diploid leaves, averaged 3.9 stomata per 100 μm^2. Here stomata density was significantly higher than that on autotetraploid leaves with average 1.6 stomata per 100 μm^2. The reduced number of stomata cells on autotetraploid leaves was compensated for with increased size per stoma and stomata guard cells (Fig. 14).

Table 10. *Stomata density on diploid and tetraploid T. vulgaris leaves*

Variants	Stomata density
Diploid	13.66 ± 2.48^{a}
Tetraploid	6.24 ± 1.26^{b}

[a,b,c] Means within the same row with different superscripts differ ($P < 0.05$ – $P < 0.001$).

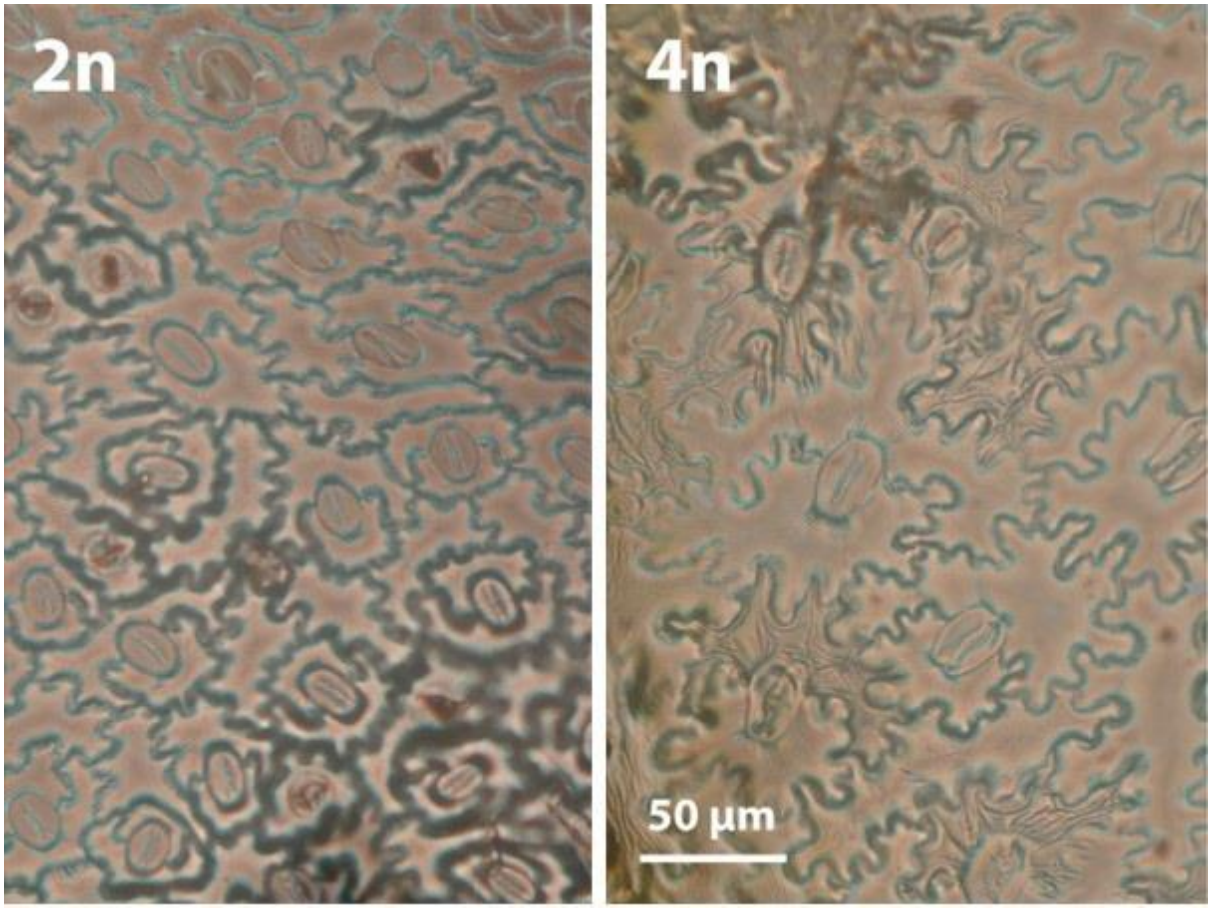

Figure 14. Stomata and stomata guard cells on the abaxial side of autotetraploid and diploid *T. vulgaris*

6.1.5.3. Glandular trichome anatomy

The distribution of diploid and autotetraploid *T. vulgaris* glandular trichomes on the leaves is random. The number of glandular trichomes is higher on diploid leaves (minimum of 16 glandular trichomes per mm^2) compared to the tetraploid leaves (minimum of 10 glandular trichomes per mm^2), on the adaxial side of expanded leaves (Figures 15 and 16). Observations also showed larger size and darker yellow colour of trichomes on tetraploid leaves.

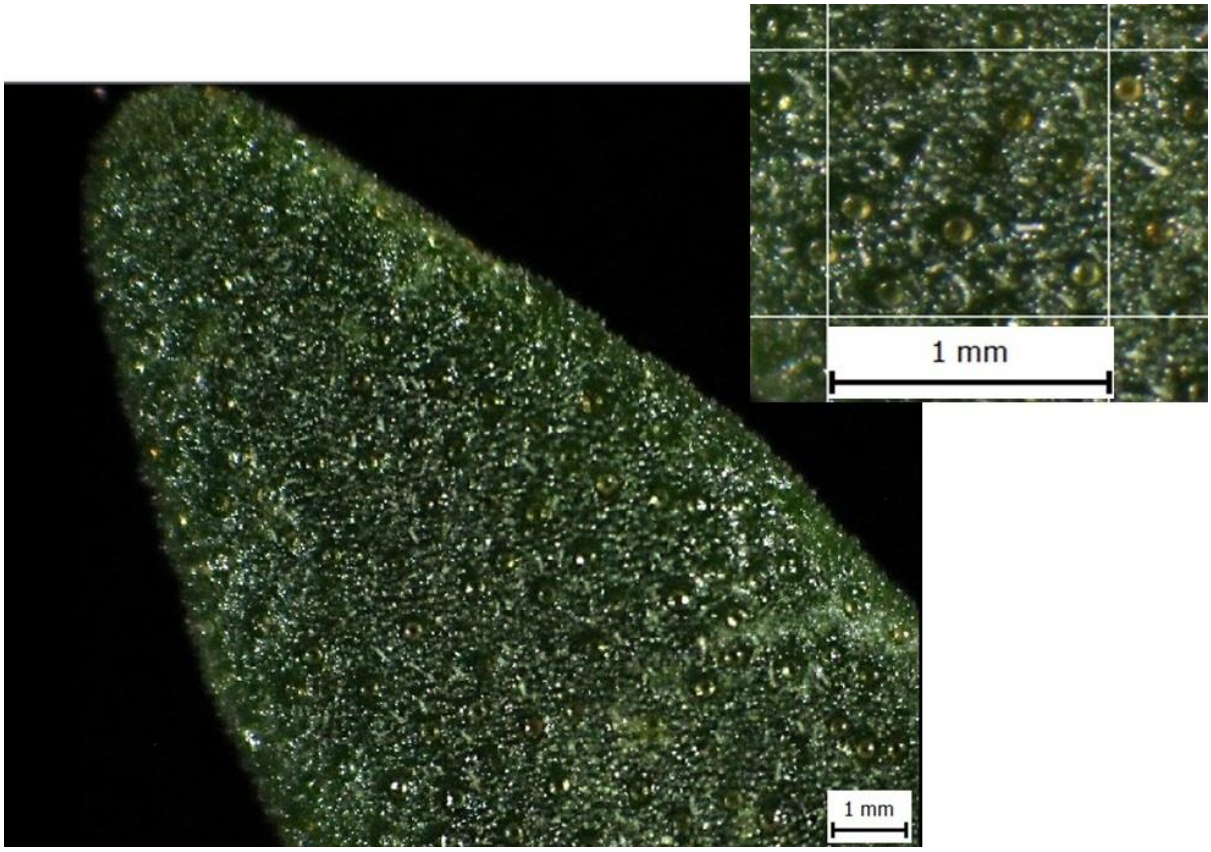

Figure 15. Autotetraploid *T. vulgaris* glandular trichomes on the adaxial leaf surface

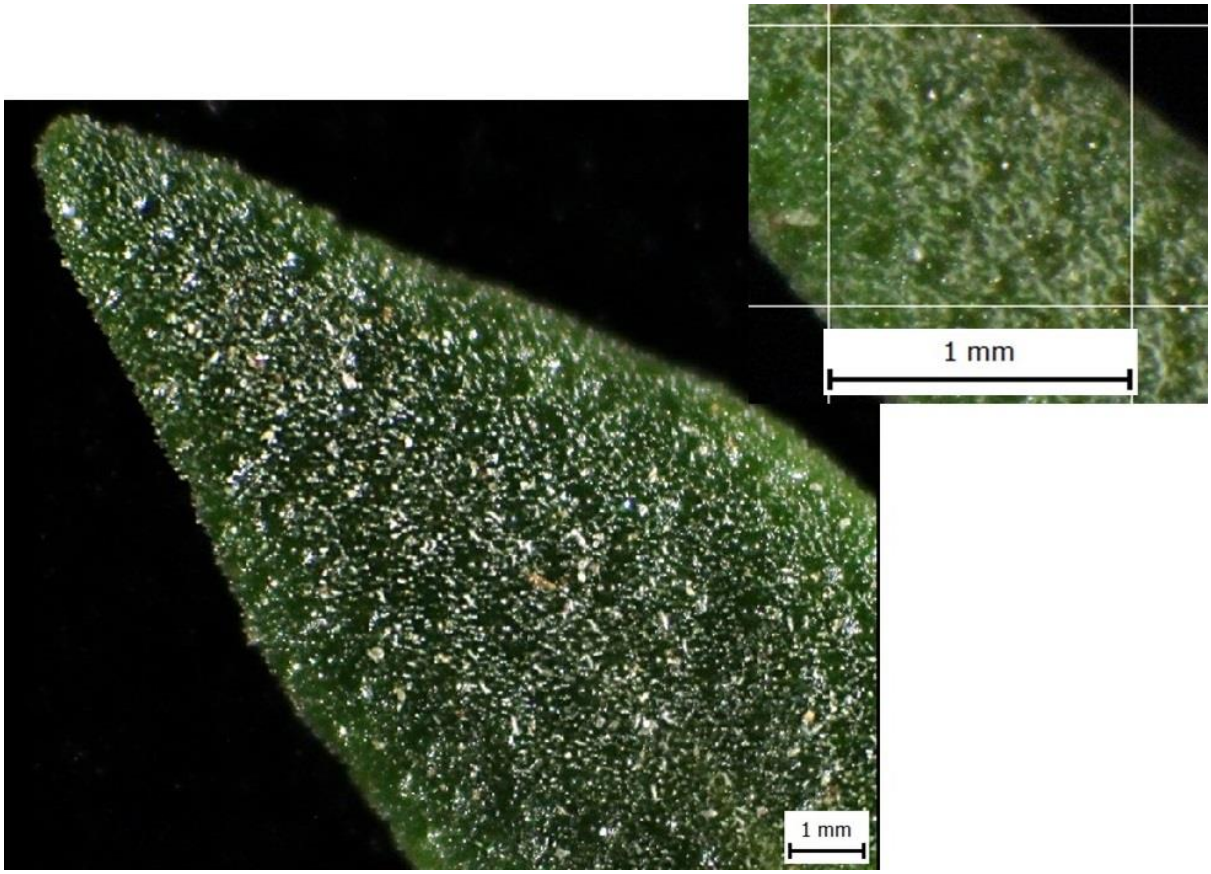

Figure 16. Diploid *T. vulgaris* glandular trichomes on the adaxial leaf surface

6.1.6. Physiological variation between diploid and tetraploid *T. vulgaris*

6.1.6.1. Chlorophyl a and b content

Successful polyploidy induction resulted in noticeable leaf colour difference between darker green colour of autotetraploid plants and their diploid controls suggesting variation in chlorophyll contents. To validate this, chlorophyll a and chlorophyll b contents were measured using a spectrophytometer. Compared to the diploid plants, tetraploid plants showed higher chlorophyll a and b content and nearly doubled in amount per gram fresh weight (Fig. 17). Chlorophyll a increased from 0.44 mg/g-1 FW to 0.76 mg/g-1 FW and chlorophyll b increased from 0.153 mg/g-1 FW to 0.249 mg/g-1 FW.

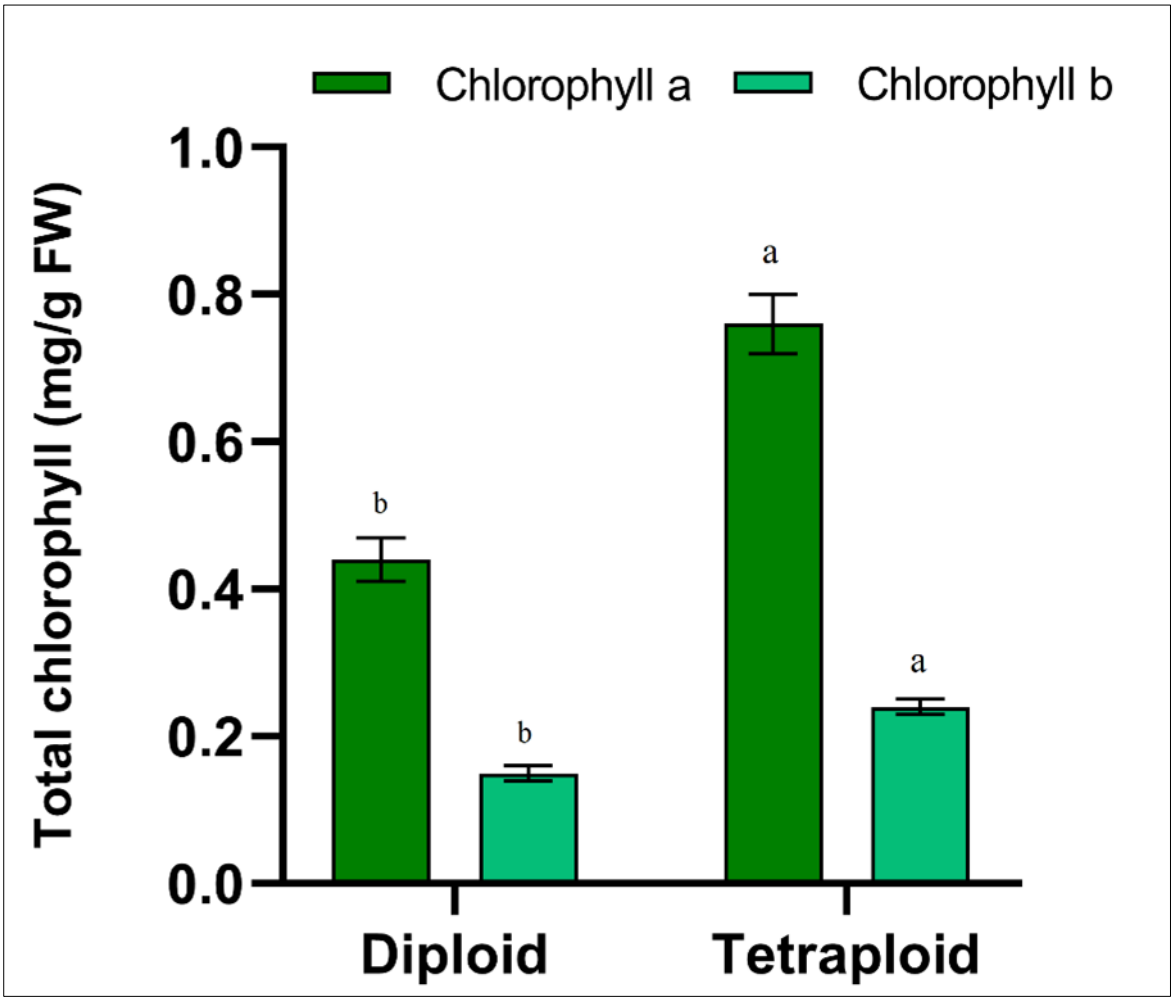

Figure 17. Chlorophyll a and b content comparison between diploid and tetraploid *T. vulgaris*

6.2. *Satureja montana* L.

6.2.1. Polyploidization efficiency

The survival rate of 100 oryzalin-treated plants was in total 6 %, in concentration of 20 μM. The regeneration percentage was not affected by the exposure duration, with same percentage in the 24 h group and in the 48 h group (Tab. 11).

Table 11. Effect of *in vitro* oryzalin treatment on the survival rate and number of polyploids in *S. montana*

Oryzalin concentration (μM)	No. of explants treated (nodal segments)	Treatment duration (h)	Survival rate (%)	No. of tetraploid plants	Polyploidization efficiency (%)
0	50	24	100	0	0
	50	48	100	0	0
20	50	24	6	3	6
	50	48	6	3	6
Total	200			6	

In total, from the 100 influenced plants, 3 % of autotetraploid genotypes were obtained in concentration 20 μM/24h (Poly 1, Poly 2 and Poly 3). And 3 % of autotetraploid genotypes were obtained in concentration 20 μM/48h (Poly 4, Poly 5 and Poly 6).

6.2.2. Flow cytometry analysis

Detection of ploidy level was carried out 3 months after ploidy induction using flow cytometry analysis. Relative DNA content of diploid *S. montana* (2n = 2x = 30) was set to channel 200 (Fig. 18) and constant parameters were maintained to follow measurements of influenced plants.

On the second histogram (Fig. 19), the first *in vitro* induced autotetraploid genotype of *S. montana* (2n = 4x = 60) was detected with relative DNA content on channel 400.

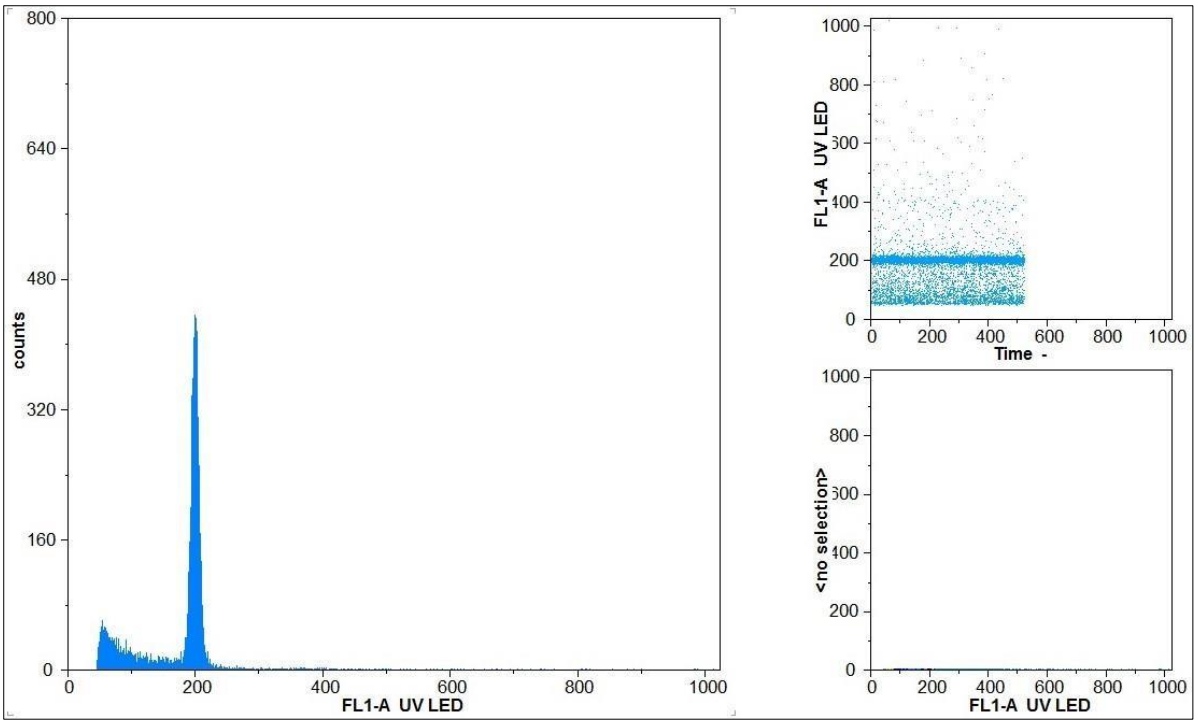

Figure 18. Histogram of relative DNA content with a peak corresponding to G0/G1 nuclei of the control plant on Channel 200

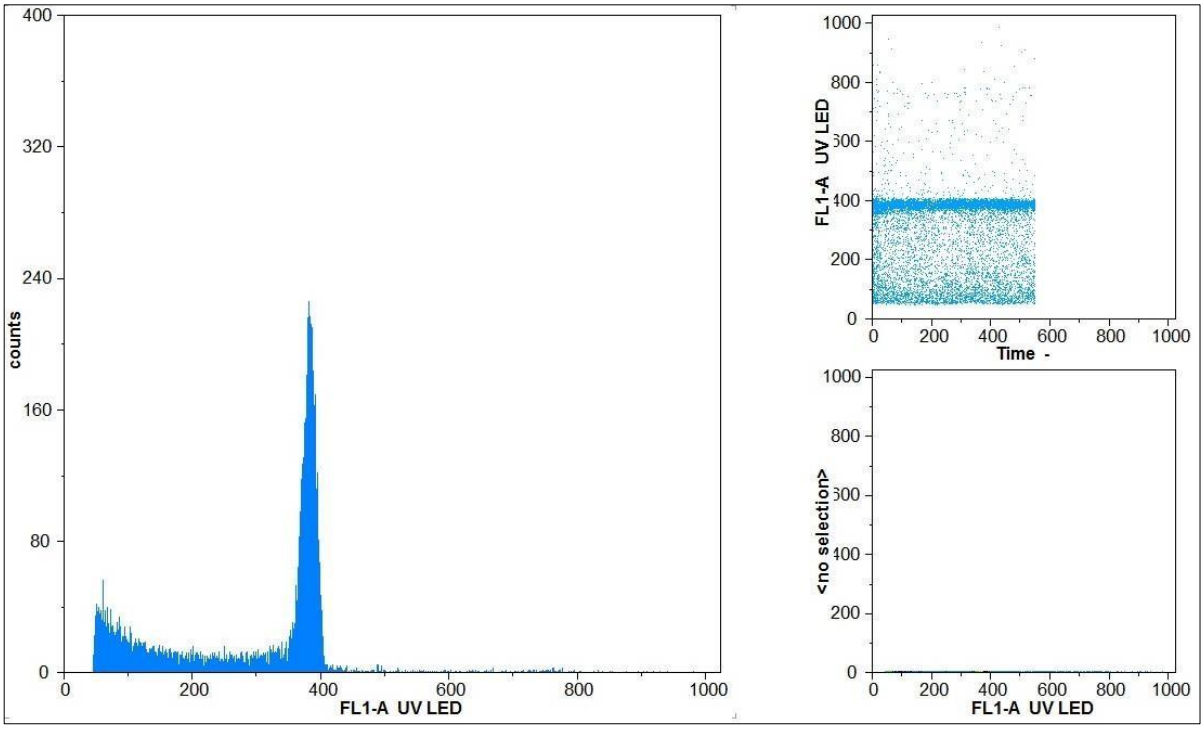

Figure 19. Histogram of relative DNA content with a peak corresponding to G0/G1 nuclei of tetraploid plant on Channel 400

Six plants survived the ploidy induction, and each of these six plants was confirmed to be an autotetraploid. Plants with well-developed root systems and healthy vigour were further

multiplied in preparation for *ex vitro* transfer. A total of 70 plants, 10 tetraploids of the six genotypes and 10 control diploid plants, were transferred to *ex vitro* conditions. The conversion survival rate of plants in *ex vitro* conditions was 0 % in tetraploid and control diploid plants.

6.2.3. *In vitro* morphological variation between diploid and tetraploid *S. montana*

In vitro morphological analysis showed growth rate variations between each individual autotetraploid. In addition, growth rate varied between the autotetraploid genotypes compared to the diploid control plants. Figure 20 shows the difference in nodal segment formation among the six obtained genotypes. Counting the number of new nodes, the control variant prevailed, however, autotetraploid genotypes Poly 4 and Poly 6 showed good nodal segment growth as well.

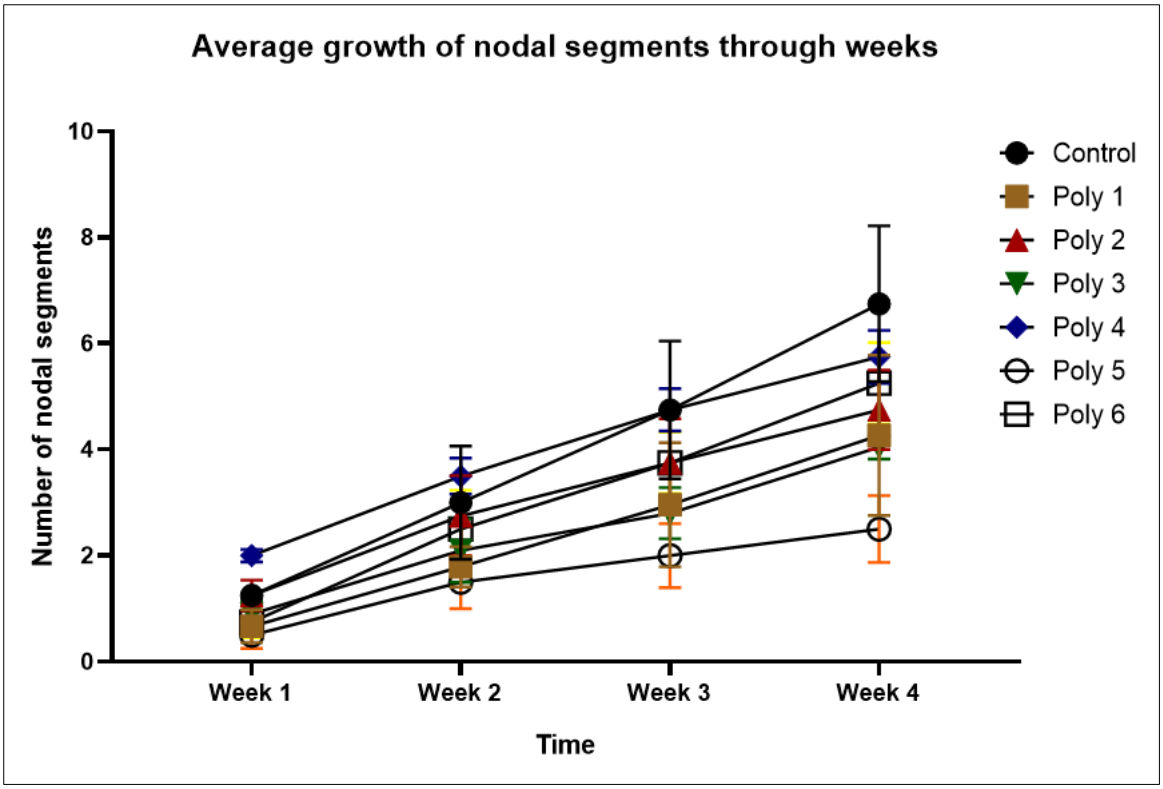

Figure 20. Average *in vitro* growth of nodal segments along one month

Genotype Poly 5 did not develop roots and exhibited overall poor growth. Autotetraploid genotype Poly 6 was late in root development. Control diploid plants and autotetraploid genotype Poly 1 were the first to develop healthy root systems in the second week following polyploidization. As all plants did not survive the initial *ex vitro* transfer trial, therefore, no *ex vitro* morphological analysis is available at this point. *In vitro* propagation of control diploids and obtained autotetraploid genotypes of *S. montana* continues in preparation for the *ex vitro* transfer stage.

7. Discussion

7.1. *Thymus vulgaris* L.

7.1.1. Oryzalin induced polyploidization

In vitro polyploidization using different microtubule inhibitors is a rapid method for obtaining polyploid plants with enhanced features. This requires the performance of test series in order to determine the most effective antimitotic agent, its concentration, and treatment duration (Dhooghe et al. 2011). The survival percentage of *T. vulgaris* obtained in this study is in agreement with previously published data for other Lamiaceae species, where (1) the survival rate of *Pogostemon cablin* increased from 9.52 % to 66.67 % with the decrease in colchicine concentration (Wu & Li 2013) and (2) the regeneration percentage of *Scutellaria viscidula* decreased from 83.3 % to 13.3 % with increasing treatment time from 6 to 36 hours (Huang et al. 2014). However, only the highest intensity of oryzalin treatment resulted in the acquisition of polyploid individuals of *T. vulgaris* individuals; whereas the concentration-dependent polyploidization efficiency varies in other studies within Lamiaceae species (Tavan et al. 2015; Wu & Li 2013; Yavari et al. 2011). The formation of a root system is of high importance in the *ex vitro* transfer stage. According to Tavan et al. (2015), *T. persicus tetraploid* plants have shown a delay in rooting compared to control diploids, while tetraploid *T. vulgaris* plants did not show a difference in growth rate compared to control diploids in our study. Our results account for the first *in vitro*-induced polyploidization of *T. vulgaris* using oryzalin. Polyploidy has recently also been successfully induced in a related species, *T. persicus*, using colchicine (Tavan et al. 2015). Colchicine is still the most preferred agent for polyploidy induction (Salma et al. 2017; Eng & Ho 2019). However, because it binds poorly to plant tubulins, it must be used in relatively high concentrations. This requires relatively large amounts of an expensive chemical (Klíma et al. 2008), while oryzalin is low at cost (Viehmannová et al. 2009). Nevertheless, oryzalin can be more effective and results in better plant quality than colchicine in some cases (Geoffriau et al. 1997; Greplova et al. 2009; Gallone et al. 2014), probably due to higher oryzalin affinity in binding to plant microtubules (Allum et al. 2007; Dhooghe et al. 2011). These drawbacks of colchicine encourage a quest for alternative antimitotic inhibitors (Dhooghe et al. 2011). This is seen in several experiments using colchicine and comparing it to the polyploidization effects of other antimitotic agents such as oryzalin, trifluralin and pendimethalin (Klíma et al. 2008; Viehmannová et al. 2009; Liu et al. 2022; Sabooni et al. 2022; Shi et al. 2022; Wen et al. 2022; Fakhrzad et al. 2023).

7.1.2. Polyploidization effect on quantitative and morphological parameters

As mentioned before, the outcome of polyploidization is often clear in morphological traits where synthesized plants exhibit enlarged cells and organs (Zhang et al. 2010). Morphological parameters were evaluated in 2019 for plants growing under greenhouse conditions and reaffirmed for autotetraploid and diploid *T. vulgaris* plants growing under field conditions. Due to genomic DNA duplication, the autopolyploid *T. vulgaris* displayed different agronomic traits from their diploid counterparts while the main statistically significant differences were recorded in leaf parameters and growth vigour of the diploid and tetraploid thyme.

Similarly, changes in morphology have previously been observed, e.g., in *Scutellaria viscidula* with tetraploids producing larger roots, stems and leaves compared to diploids (Huang et al. 2014). And in *Tetradenia riparia*, where tetraploid plants had thicker leaves and more rounded and highly lobed leaf margins compared to diploids. Similarly, autotetraploid Chinese jujube (*Ziziphus jujuba*) increased leaf width by 52.83 % compared to the diploid from which it was induced (Wang et al. 2019). Liu et al. (2019) studied artificial triploid Loquat (*Eriobotrya japonica*) which had larger and greener leaves along with more vigorous growth than their diploid and tetraploid progenitors. Contrary to our results, Tavan et al. (2015) has previously reported that *T. persicus* plants had significantly shorter internode lengths and overall plant heights compared to diploids, which could be caused by the delay in rooting of tetraploid plants.

7.1.3. Polyploidization effect on biochemical content

Both diploid and tetraploid *T. vulgaris* plants contained larger quantities of thymol compared to other essential oil components. Essential oil of *T. vulgaris* can have different chemotypes including thymol, linalool, carvacrol, thujanol-4, geraniol and terpineol (Özcan & Chalchat, 2004; Boaventura et al. 2022). Chemical analysis of *Thymus vulgaris* showed a thymol content of 30.31 % and 48.32 % in year 2019, and repeated analysis for plants growing on field conditions showed a thymol content of 29.33 % and 50.12 % in essential oil of diploid and tetraploid, respectively. According to a recently described thymol biosynthetic pathway in thyme (Krause et al. 2021), γ-terpinene is first bio-synthesized from geranyl diphosphate and then serves as a precursor for the synthesis of all thymol, carvacrol and p-cymene via several intermediates. There is also little difference in the sum content of all these four main components in EO from diploid (78.59 %) and tetraploid (80.09 %) plants.

The second essential oil analysis performed for plants growing on field conditions showed 20.79 % higher thymol content in autoteraploid *T. vulgaris* plants. Accordingly, Noori et al.

(2017) reported almost 20 % higher thymol content in the essential oil derived from tetraploid *Trachyspermum ammi* seeds. The increase in essential oil content in tetraploid individuals has been previously observed also in other species belonging to Lamiaceae family (Yavari et al. 2011; Wu and Li, 2013; Hannweg et al. 2016b) as well as to other families of aromatic plants (Iannicelli et al. 2016; Tsuro et al. 2016; Noori et al. 2017).

The increase in a certain metabolite, whether primary or secondary, is compensated by the decrease in another primary or secondary metabolite. In other words, activation of a certain gene will lead to the expression of its corresponding phenotype. This activation is sometimes parallel to the silencing of another gene, which in turn will tie down or eliminate its corresponding phenotype. We mentioned the increase in thymol in the second essential oil analysis, but in the same analysis, p-cymene decreased from 32.11 % in diploid *T. vulgaris* plants to 15.33 % in tetraploid *T. vulgaris* plants (Tab. 8). This variability is observed in both primary and secondary metabolites. According to Tan et al. (2015, 2017, 2019), secondary metabolites such as flavones, phenylpropanoids and terpenoids tend to decrease in favour of increased production of primary metabolites in polyploid plants of *Citrus junos*, *C. reticulata,* and *Poncirus trifoliata*. It has been assumed that the primary metabolism enhancement in polyploid plants probably helps to relieve genomic stress and to promote vitality and growth.

According to the antioxidant activity analyses, we obtained significantly increased total phenol and flavonoid contents in *T. vulgaris* autotetraploid plants compared to their diploid progeny plants. Similarly induced polyploid *Salvia officinalis* L. plants showed an increase in phenolic, flavonoid, and total antioxidant activities, among other plant physiological processes (Hassanzadeh et al. 2020). In five antioxidant test settings, tetraploid essential oil of *Citrus limon* (L.) Osbeck had enhanced antioxidant activity. Here increased monoterpene content in induced tetraploid plants could be the reason behind their improved antioxidant capacity (Bhuvaneswari et al. 2020). Higher content of the total phenolic acid and monomeric compounds in tetraploid *Echinacea purpurea* (L.) plants is related to higher antioxidant activity compared to diploid plants (Mei et al. 2020). As for autopolyploid *T. vulgaris*, essential oil analysis showed main increase in monoterpenoid phenols, and in line with the mentioned studies, the autotetraploid *T. vulgaris* genotype was superior in its antioxidant activity to the diploid control in DPPH, ORAC, TEAC, TPC and TFC analyses. On the contrary, the total phenol content analysis for several diploid and tetraploid samples of *Gynostemma pentaphyllum* (Thunb.) Makino plants revealed highest phenol content for diploid samples. Interestingly, the same diploid samples also expressed the highest flavonoid content and

antioxidant activity (Xie et al. 2012). Considering the common variability in essential oil composition, even within a single species, it is not surprising that not only the essential oil yield but also its volatile profile and concentration of active constituents is affected by polyploidization (Sattler et al. 2016).

7.1.4. Polyploidization effect on anatomical features

7.1.4.1. Quantitative wood anatomy

Variability in anatomical features of the xylem in shrubs and herbaceous species, addresses research questions related to plant functioning, growth and environment (von Arx et al. 2016). A stronger xylem system with larger vessels in tetraploids compared with their respective diploid parental lines could transport water and nutrients more efficiently (Marfil et al. 2018). The most distinctive xylem cells, the tracheid cells, were significantly higher in number in autotetraploid *T. vulgaris* plants. Furthermore, the autotetraploid genotype had larger cell lumen compared to diploid control plants. Oryzalin induced polyploidization of two diploid potatoe species, generated polyploid lines of *Solanum commersonii* Dunal and *Solanum bulbocastanum* Dunal (Aversano et al. 2013). Comparable to our results, the lumen area of vessel in the tetraploid *S. commersonii* was significantly lower than in its diploid control. However, contrary to our results, the lumen area of vessel in the tetraploid *S. bulbocastanum* was greater than in the diploid control.

Another significant difference in the anatomical characteristics of *T. vulgaris* was the thicker radial cell wall of diploid controls compared to autotetraploid plants. Colchicine induced tetraploids of *Manihot esculenta* Crantz presented similar results. The xylem of tetraploid plants was wider but had thinner cell walls compared to its diploid cassava controls (Nassar et al. 2008).

7.1.4.2. Stomata density

Autotetraploid *T. vulgaris* leaves had significantly lower stomata count than diploid leaves. The decrease in stomata count could be related to increased stomata size observed in tetraploid leaves. *In vitro* induced autopolyploidization in one thyme species (*Thymus persicus)*, six sage species (2x *Salvia miltiorrhiza*, *Salvia leriifolia*, 2x *Salvia officinales* and *Salvia multicaulis*) and eight other species (*Scutellaria baicalensi*, *Lavandula angustifolia*, *Ocimum basilicum*, *Dracocephalum moldavica*, *Dracocephalum kotschyi*, *Pogostemon cablin*, *Melissa officinalis* and *Mentha* × *villosa*) from the Lamiaceae family showed the same results when comparing autopolyploid plants to their diploid progenitors. In all of the aforementioned species, stomata

cell size increased and stomata density decreased in autopolyploid plants compared to their progenitors (Gao et al. 1996; Gao et al. 2002; Urwin et al. 2007; Omidbaigi et al. 2010a; Omidbaigi et al. 2010b; Zahedi et al. 2014; Tavan et al. 2015; Yan et al. 2016; Estaji et al. 2017; Chen et al. 2018; Talei & Fotokian 2020; Hassanzadeh et al. 2020; Tavan et al. 2021; Tavan et al. 2022; Moetamedipoor et al. 2022).

Stomata characteristics fall under the category of secondary methods for the identification of autopolyploid plants. It seems to be a standard autopolyploidization effect on stomata size and density as shown in the 15 articles above. The 'gigas effect' of polyploidy in plants is the cell enlargement which results in increased organ size. This phenotypic change is commonly observed in polyploid plants (Balao et al. 2011; Becker et al. 2022). We confirm this as we clearly see the 'gigas effect' playing its role as a major polyploidy effect.

7.1.4.3. Glandular trichome anatomy

The density of the glandular trichomes was lower in the tetraploid leaves of *T. vulgaris,* but larger in size compared to the trichomes of the diploid leaf. Furthermore, macroscopy observations of *T. vulgaris* showed that the trichomes were darker and yellowish in colour. The trichomes of the tetraploid *Lavandula angustifolia* were larger in size relative to the diploid controls, but their distribution and spacing appeared to be resembling to those of the diploids (Urwin et al. 2007). The enlarged trichome size of tetraploid *T. vulgaris* was similar to that of tetraploid *L. angustifolia.*

In the Lamiaceae family, an increase in trichome density was reported in contrast to our results in autopolyploid *Dracocephalum kotschyi, Thymus persicus,* and *Salvia officinalis* (Zahedi et al. 2014; Tavan et al. 2015; Tavan et al. 2021). While decreased trichome density in concurrence with our results was reported only in *Salvia multicaulis* (Tavan et al. 2022).

Zahedi et al. (2014) relates between the enhancement of secondary metabolites and the high glandular trichomes structure in tetraploid plants. After comparing their results between diploid and tetraploid *L. angustifolia,* Urwin et al. (2007) concluded that enhanced oil yield from the tetraploids may depend on plant size and flower number. The decrease in glandular trichome density on diploid *T. vulgaris* leaves is compensated by the increase in leaf size and the increase in size of the glandular trichomes in tetraploid *T. vulgaris* leaves. Glandular secretory trichomes are biofactories that synthesise, store and secrete monoterpenes (Yan et al. 2017). Transcriptomic analyses of *T. vulgaris* genome identify genes that play important roles in glandular secretory trichome formation and monoterpenoid biosynthesis (Sun et al. 2022).

Whole genome duplication caused changes in glandular trichome anatomy in the tetraploid *T. vulgaris* genotype. These changes were accompanied by enhanced essential oil profile and increased essential oil yield.

7.1.5. Polyploidization effect on physiological parameters

7.1.5.1. Chlorophyl a and b content

Chlorophyll concentration is a crucial indication to assess photosynthetic mechanism and plant metabolism (Kamble et al. 2015). Artificial polyploidization is known to alter chlorophyll content variably. In this study, artificial polyploidization affected the chlorophyll content significantly between the diploid and the synthetic tetraploid *T. vulgaris*. Chlorophyll a and chlorophyll b increased by 72.72 and 62.74 %, respectively. Our results align with several studies previously published research: increased levels of chlorophyll content are reported in plants including *Stevia reboudiana* (Zhang et al. 2018), *Rhododendron fortune* (Lan et al. 2020) and *Artemisia annua* (Yunus et al. 2018). Chlorophyll pigment content reflects the physiological performance in leaves and plants. The increase of chlorophyll content could influence carbohydrate metabolism by promoting photosynthesis (Lan et al. 2020). Higher chlorophyll contents could be related to increased photosynthetic rates and increased vigour of tetraploid plants (Zhang et al. 2018). Leaf colour of the autotetraploid *T. vulgaris* plants was noticeably darker than that of the diploid control plants. Increased chlorophyll contents justify the darker green colour of several induced polyploid plants (Lan et al. 2020). However, several studies have also reported decreased levels of chlorophyll content in synthetic polyploids compared to their control plants (Manzoor et al. 2018; García-García et al. 2020). This could be due structural modifications of chloroplast components (Xu et al. 2010). Overall, this indicates the variability of chlorophyll content among synthetic polyploids.

7.2. *Satureja montana* L.

7.2.1. Oryzalin induced polyploidization

The effective concentration of a certain antimitotic agent required for successful polyploidy induction depends on different experimental variables and on the experimented species. Most studies use on average four different concentrations of an antimitotic agent (Diem et al. 2022; Fakhrzad et al. 2023). Furthermore, several polyploidization studies choose two (Sabooni et al. 2022), three (Shi et al. 2022) or more (Mitrofanova et al. 2003) antimitotic agents. We chose oryzalin for the polyploidy induction of *S. montana* and applied it at a single concentration of 20 μM. Our decision was based on previous and ongoing results from laboratory experiments

with ploidy induction of several Lamiaceae species. The morphology of *in vitro* cultured *S. montana* dictated delicate manipulation during propagation. Based on the fragile character of *in vitro S. montana* cultures, while multiplying nodal segments in preparation for the experiment, it was clear that a low oryzalin concentration will be required for chromosome doubling. Liu et al. (2021a) treated the apical buds of *Fragaria nilgerrensis* with one concentration of 0.2 % colchicine gel solution. Their treatment scheme was based on a previously conducted, unpublished gradient experiment. Our polyploidization efficiency was relatively low, but we successfully obtained six genotypes of *S. montana.* Yet, the total explant viability of 6 plants from 100 healthy nodal segments is an indication that lower concentrations of oryzalin could be effective. Decreasing antimitotic concentrations can be tricky as the survival rate could increase but the polyploidization effect is compromised. Increasing oryzalin concentrations and exposure durations led to the significant reduction in *Allium sativum* viability (Wen et al. 2022). Regarding the duration of exposure to antimitotic agent, Viehmannová et al. (2009) obtained two polyploid *Smallanthus sonchifolius* genotypes from 20 μM oryzalin concentration, one from each time duration of 24 and 48 h. Exposure durations of 24 and 48h had identical effects on polyploidization efficiency and survival rate of *S. montana*.

Ex vitro transfer is a critical stage for *in vitro* plant cultures where a substantial number of plants do not survive the transfer to greenhouse conditions (Kumar & Rao 2012). Our first transfer of *S. montana* plants to *ex vitro* conditions was challenging as the plants did not survive. Micropropagation of *Satureja khuzistanica* highlighted the importance of different soils for survival rate during the acclimatization stage, and (sand:soil:peat:perlite) at a ratio of (1:1:1:1) showed the highest percentage of survival (Mirjani et al. 2018). In another study on *S. avromanica* plants were transferred and established in plastic pots containing (peat moss:coco peat:perlite) at a ratio of (1:1:1) (Mozafari et al. 2015). We used a similar mixture of (sand:soil:peat moss:vermiculite) at a ratio of (1:1:1:1;v/v) in our experiment. Several variables affect the success of the *ex vitro* transfer stage. One such important variable responsible for successful acclimatization is humidity. In a successful *ex vitro* transfer of *Satureja avromanica*, out of 100 transferred plants, 60 survived (Mozafari et al. 2015). These *S. avromanica* plants were covered with polyethylene bags to maintain humidity. Polyethylene bags were also used in the acclimatization of *Satureja abyssinica* plants and a high survival rate of 88 % was achieved (Teshome et al. 2017). Sarropoulou and Maloupa (2019) reported a similar survival rate of 81.82 % for *Satureja thymbra* plants. Acclimatization of *S. thymbra* plants included using a mist system of an unheated greenhouse for maintaining humidity.

Gradually decreasing air humidity during acclimatization is the most important factor for effective stomatal regulation of transpiration, leading to stabilization of water status (Pospíšilová et al. 2007). Development of cuticle, epicuticular waxes and functional stomatal apparatus, are other major challenges during transfer *ex vitro* conditions (Pospíšilová et al. 2007).

7.2.2. Polyploidization effect on *in vitro* morphological parameters

Morphological characteristics are easily identifiable and can be used to preliminarily identify polyploid plants. Increased ploidy level causes obvious morphological changes in polyploid plants (Mo et al. 2020).

We observed and measured growth rate variations between autotetraploid genotypes of *S. montana* and their diploid controls. Nodal segment growth prevailed in the diploid controls and thus diploids grew longer and larger than the autotetraploids. Similar results were published by Ghanbari et al. (2019) in tetraploid *Impatiens walleriana* plants which had a lower growth rate compared to their diploid counterparts.

Each one of the six *S. montana* autotetraploids was variable in segment formation and overall growth character. In contrast, Wen et al. (2022) exhibited stable and uniform dwarf characteristics among all autotetraploid *Allium sativum* genotypes successively cultured for more than 2 years.

Root development is crucial in the *in vitro* stage for culture proliferation. Poly 5 did not develop a root system and Poly 6 was relatively late in root development. Comparing all the autotetraploid genotypes to diploid controls, root development was faster in diploid plants. Tavan et al. (2021) reports lower height of *in vitro* polyploid *Salvia officinalis* plants compared to diploid counterparts due to reduced root growth. Plant Growth Regulators (PGRs) were used for initial root proliferation in *S. officinalis*, eventually control plants and regenerants were sub-cultured on MS medium free of PGRs. We did not use any PGRs to maintain certainty that any monitored change is a result of and caused only by the polyploidization procedure. PGRs have been reported to interact with antimitotic agents, for example, adding Indole-3-acetic acid to media enhanced polyploidization of *Acer platanoides* by indirectly leading to an increase in sensitivity to oryzalin (Lattier et al. 2013). While addition of 6-benzylaminopurine to media increased shoot mortality of *A. platanoides* at higher oryzalin concentrations regardless of exposure duration.

As a valuable medicinal plant, polyploidization provides new genetic material for future breeding programs of *S. montana*. We introduce a new protocol for *in vitro* autopolyploid induction of winter savory using oryzalin and without using PGRs.

8. Conclusion

In this study a new protocol for the *in vitro* polyploidization of *Thymus vulgaris* and *Satureja montana* is proposed using oryzalin as an antimitotic agent. Ploidy levels of one autotetraploid genotype of *T. vulgaris* and six genotypes of *S. montana* were verified by flow cytometric analysis. We confirm the first hypothesis for the effectivity of oryzalin as an antimitotic agent for ploidy induction in both plant species. And we confirm the second hypothesis as different ploidy levels were measured between control plants and autopolyploid genotypes. This is the first published autotetraploid genotype of *T. vulgaris* obtained via *in vitro* polyploidization. To the best of our knowledge, *in vitro* polyploidization has not been attempted so far on *S. montana*.

The 'gigas effect' of polyploidization on the vigorous growth of autotetraploid *T. vulgaris* plants was translated into increased fresh and dry plant weight. *In vitro* morphological characteristics of autotetraploid plants of *S. montana* are promising and the biochemical analyses of this medicinal plant has yet to be evaluated.

The study demonstrates the effect of polyploidization on the biochemical profile of the plant where the new genotype of *T. vulgaris* expressed qualitative and quantitative enhancements; the yield of essential oil tetraploids increased from 0.81 % to 1.28 % and the content of several main components increased, including thymol and carvacrol by 20.79 % and 0.98 %, respectively. The qualities of the new *T. vulgaris* autotetraploid improve an already rich culinary herb and medicinal plant.

Morphological, biochemical, anatomical and physiological analyses performed in this study are valuable results for plant biotechnology research, are in harmony with other polyploidization experiments, and prove that the overall result of any induced autopolyploidization experiment is variable. This also confirms the third hypothesis for differences in the mentioned analyses between control plants and autopolyploid genotypes.

The introduction of *T. vulgaris* as a reliable autopolyploid genotype for commercial production depends on its genetic stability. Autopolyploid genotypes of *T. vulgaris* are genetically stable after growing in *ex vitro* conditions confirming the fourth hypothesis of this study. The success of polyploidization is proven by repeated flow cytometric analysis during two years of growing under field conditions. This stability is further expressed in repeated biochemical analyses for the essential oil content of this medicinal plant.

Future breeding prospects

The new genetic material of *T. vulgaris* and that of *S. montana* play an important role in the new era of sustainable development as we gradually replace synthetic chemicals with naturally occurring products. The green revolution enabled us to increase our agricultural production and solved the food security problems of the past. Today, we have the tools to continue to progress, but we need to utilise our natural resources to maintain sustainability. This research produced a medicinal plant with an enhanced chemical profile and a higher essential oil content. This is important to address the increased demand in recent years caused by; demographical changes caused by wars which increased the picking of medicinal plants from the wild. Increased demand for the commercial production of components of essential oils for the production of pharmaceuticals. The recent boom in the use of essential oil from medicinal plants in agrochemicals.

Autopolyploidization of more than 20 plant species between the years 2021 and early 2023 shows the interest of the scientific platform to supply the increased demand for different plant species. Different components of *T. vulgaris* and *S. montana* are currently being investigated in different fields. The need for increased production of medicinal plants will continue to increase with this scientific progress.

T. vulgaris is more expensive than other species, so the herbal products industry frequently uses other cheaper species of Thyme (Silva et al. 2021). Furthermore, the thymol chemotype is one of the most used commercially and we present an improved genotype of *T. vulgaris* with high thymol content. Although, agrotechnical studies aim to increase productivity per cultivated area. Breeding methods are crucial for yield gains and quality of the product. The increase in essential oil production per plant should be an intriguing factor for scientific disciplines to investigate these activities. The novel bio-chemical profile is yet to be tested and evaluated for its biological activities. Other agronomic characteristics of autotetraploid garden thyme such as tolerance/resistance to biotic and abiotic stress will be assessed to further express its possible advantages.

Which sector will benefit the most from autotetraploid *T. vulgaris* and *S. montana*? Will the unique EO profile of each genotype be used as a whole, or will the plants be used for the commercial production of a certain compound? What hidden plant properties will polyploidization reveal in the future? What we know now is that there is potential both in quality and in quantity.

9. References

Abdolahi A, Hassani A, Ghuosta Y, Bernousi I, Meshkatalsadat MH. 2010. *In vitro* efficacy of four plant essential oils against *Botrytis cinerea* Pers.: Fr. and *Mucor piriformis* A. Fischer. Journal of Essential Oil Bearing Plants **13**(1):97-107.

Abdolinejad R, Shekafandeh A, Jowkar A. 2021. *In vitro* tetraploidy induction creates enhancements in morphological, physiological and phytochemical characteristics in the fig tree (*Ficus carica* L.). Plant Physiology and Biochemistry **166**:191-202.

Aćimović M, Šovljanski O, Pezo L, Travičić V, Tomić A, Zheljazkov VD, Ćetković G, Švarc-Gajić J, Brezo-Borjan T, Sofrenić I. 2022. Variability in biological activities of *Satureja montana* subsp. *montana* and subsp. *variegata* based on different extraction methods. Antibiotics **11**(9):1235.

Adams RP. 2007. Identification of essential oil components by gas chromatography/mass spectrometry. fourth ed. Allured Publishing Corporation, Illinois, USA.

Afonso S, Oliveira I, Ribeiro C, Vilela A, Meyer AS, Gonçalvesa B. 2023. Innovative edible coatings for postharvest storage of sweet cherries. Scientia Horticulturae **310**:111738.

Allum JF, Bringloe DH, Roberts AV. 2007. Chromosome doubling in a *Rosa rugosa* Thunb. hybrid by exposure of *in vitro* nodes to oryzalin: the effects of node length, oryzalin concentration and exposure time. Plant Cell Reports **26**:1977-1984.

AL-Hajri MZM, Hossain MA, AL-Touby SS. 2022. Composition analysis and antibacterial activity evaluation of different crude extracts of *Mentha piperita* (Lamiaceae). International Journal of Secondary Metabolite **9**(4).

Al-Ramahi R, Jaradat N, Adawi D. 2013. Use of herbal medicines during pregnancy in a group of Palestinian women. Journal of Ethnopharmacology **150**:79-84.

Al-Ramamneh EAl-DM. 2009. Plant growth strategies of *Thymus vulgaris* L. in response to population density. Industrial Crops and Products **30**:389-394.

Alirezalu K, Pateiro M, Yaghoubi M, Alirezalu A, Peighambardoust SH, Lorenzo JM. 2020. Phytochemical constituents, advanced extraction technologies and techno-functional properties of selected Mediterranean plants for use in meat products. A comprehensive review. Trends Food Science and Technology **100**:292-306.

Amanlou M, Dadkhah F, Salehnia A, Farsam H, Dehpour AR. 2005. An anti-inflammatory and anti-nociceptive effect of hydroalcoholic extract of *Satureja khuzistanica* Jamzad extract. Journal of Pharmacy and Pharmaceutical Sciences **8**(1):102-106.

APG IV, The Angiosperm Phylogeny Group. Chase MW, Christenhusz MJM, Fay MF, Byng JW, Judd WS, Soltis DE, Mabberley DJ, Sennikov AN, Soltis PS, Stevens PF. 2016. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV. Botanical Journal of the Linnean Society **181**(1):1-20.

Arnold B, Kim ST, Bomblies K. 2015. Single geographic origin of a widespread autotetraploid *Arabidopsis arenosa* lineage followed by interploidy admixture. Molecular Biology and Evolution **32**(6):1382-1395.

Askary M, Behdani MA, Parsa S, Mahmoodi S, Jamialahmadi M. 2018. Water stress and manure application affect the quantity and quality of essential oil of *Thymus daenensis* and *Thymus vulgaris*. Industrial Crops and Products **111**:336-344.

Aversano R, Caruso I, Aronne G, De Micco V, Scognamiglio N, Carputo D. 2013. Stochastic changes affect *Solanum* wild species following autopolyploidization. Journal of Experimental Botany **64**(2):625-635.

Badi HN, Yazdani D, Ali SM, Nazari F. 2004. Effects of spacing and harvesting time on herbage yield and quality/quantity of oil in thyme, *Thymus vulgaris* L. Industrial Crops and Products **19**:231-236.

Bakkali F, Averbeck S, Averbeck D, Idaomar M. 2008. Biological effects of essential oils - A review. Food and Chemical Toxicology **46**:446-475.

Balao F, Herrera J, Talavera S. 2011. Phenotypic consequences of polyploidy and genome size at the microevolutionary scale: a multivariate morphological approach. New Phytologist **192**:256-265.

Becker FW, Oberlander K, Trávníček P, Dreyer LL. 2022. Inconsistent expression of the gigas effect in polyploid Oxalis. American Journal of Botany **109**(10):1-15.

Beranová K, Bharati R, Žiarovská J, Bilčíková J, Hamouzová K, Klíma M, Fernández-Cusimamani E. 2022. Morphological, cytological, and molecular comparison between diploid and induced autotetraploids of *Callisia fragrans* (Lindl.) Woodson. Agronomy **12**(10):2520.

Bezbradica DI, Tomovic JM, Vukasinovic MS, Siler-Marinkovic S, Ristic MM. 2005. Composition and antimicrobial activity of essential oil of *Satureja montana* L. collected in Serbia and Montenegro. Journal of Essential Oil Research **17**(4):462-465.

Bhardwaj R, Sharma K, Sharma DK, Parkash P. 2020. Conventional genetic improvement methods in medicinal and aromatic plants: A Review. International Journal of Economic Plants **7**(4):156-161.

Bhuvaneswari G, Thirugnanasampandan R, Gogulramnath M. 2020. Effect of colchicine induced tetraploidy on morphology, cytology, essential oil composition, gene expression and antioxidant activity of *Citrus limon* (L.) Osbeck. Physiology and Molecular Biology of Plants **26**:271-279.

Boaventura TP, dos Santos FAC, Souza AS, Batista FS, Julio GSC, Luz RK. 2022. Thymol and linalool chemotypes of the essential oil of *Thymus vulgaris* (thyme) as anesthetic for *Colossoma macropomum*: physiology and feed consumption. Aquaculture **554**:738161.

Bolechowski A, Moral R, Bustamante MA, Bartual J, Paredes C, Pérez-Murcia MD, Carbonell-Barrachina AA. 2015. Winery–distillery composts as partial substitutes of traditional growing media: Effect on the volatile composition of thyme essential oils. Scientia Horticulturae **193**:69-76.

Bomblies K, Jones G, Franklin C, Zickler D, Kleckner N. 2016. The challenge of evolving stable polyploidy: could an increase in 'crossover interference distance' play a central role? Chromosoma **300**:287-300.

Boscaiu M, Riera J, Estrelles E, Güemes J. 2000. Números cromosomáticos de plantas occidetales. Anales del Jardin Botanico de Madrid **58**:163-164.

Bojovi´c D, Šoški´c M, Tadi´c V. 2018. Comparative study of chemical composition of the essential oils from *Satureja cuneifolia* Ten. and *Satureja montana* L., Lamiaceae collected at national park Lov´cen, Montenegro. Studia Universitatis Babes-Bolyai Chemia **63**:167-180.

Bhattarai K, Kareem A, Deng ZA. 2021. *In vivo* induction and characterization of polyploids in gerbera daisy. Scientia Horticulturae **282**:110054.

Brownfield L, Köhler C. 2011. Unreduced gamete formation in plants: mechanisms and prospects. Journal of Experimental Botany **62**(5):1659-1668.

Butnariu M, Quispe C, Herrera-Bravo J, Helon P, Kukula-Koch W, López V, Les F, Vergara CV, Zapata PA, Zapata BA, Martorell M, Pentea M, Dragunescu AA, Samfira L, Yessimsiitova Z, Dastan SD, Castillo CMS, Roberts TH, Rad JS, Koch W, Cho WC. 2022. The effects of thymoquinone on pancreatic cancer: Evidence from preclinical studies. Biomedical Journal **153**:13364.

Cao G, Prior RL. 1998. Measurement of oxygen radical absorbance capacity in biological samples. Methods in Enzymology **299**:50-62.

Caprioli G, Lupidi G, Maggi F. 2019. Comparison of chemical composition and antioxidant activities of two Winter savory subspecies (*Satureja montana* subsp. *variegata* and

Satureja montana subsp. *montana*) cultivated in Northern Italy. Natural Product Research **33**(21):3143-3147.

Catani L, Grassi E, di Montanara AC, Guidi L, Sandulli R, Manachini B, Semprucci F. 2022. Essential oils and their applications in agriculture and agricultural products: A literature analysis through VOSviewer. Biocatalysis and Agricultural Biotechnology **45**:102502.

Ćavar S, Šolić ME, Maksimović M. 2013. Chemical composition and antioxidant activity of two *Satureja* species from Mt. Biokovo. Botanica serbica **37**(2):159-165.

Chen EG, Tsai KL, Chung HH, Chen JT. 2018. Chromosome doubling-enhanced biomass and dihydrotanshinone I production in *Salvia miltiorrhiza*, a traditional Chinese medicinal plant. Molecules **23**(12):3106.

Chorianopoulos N, Kalpoutzakis E, Aligiannis N, Mitaku S, Nychas GJ, Haroutounian SA. 2004. Essential oils of *Satureja*, *Origanum*, and *Thymus* species: Chemical composition and antibacterial activities against foodborne pathogens. Journal of Agricultural and Food Chemistry **52**:8261-8267.

Christ B, Müller KH. 1960. On the serial determination of the content of flavanol derivatives in drugs. Archiv der Pharmazie **293**:1033-1042.

Cigliano RA, Sanseverino W, Cremona G, Consiglio FM, Conicella C. 2011. Evolution of Parallel Spindles Like genes in plants and highlight of unique domain architecture. BMC Evolutionary Biology **11**:78.

Cui H, Wang Y, Li C, Chen X, Lin L. 2021. Antibacterial efficacy of *Satureja montana* L. essential oil encapsulated in methyl-β-cyclodextrin/soy soluble polysaccharide hydrogel and its assessment as meat preservative. Food Science and Technology **152**:112427.

De Almeida JM, Crippa BL, de Souza VVMA, Alonso VPP, Júnior EMS, Picone CSF, Prata AS, Silva NCC. 2023. Antimicrobial action of oregano, thyme, clove, cinnamon and black pepper essential oils free and encapsulated against foodborne pathogens. Food Control **144**:109356.

De Rojas VS, Somoza B, Ortega T, Villar AM. 1996. Isolation of vasodilatory active flavonoids from the traditional remedy *Satureja obovata*. Planta medica **62**(03):272-274.

De Storme N, Geelen D. 2013. Sexual polyploidization in plants – cytological mechanisms and molecular regulation. New Phytologist **198**(3):670-684.

Dhooghe E, Van Laere K, Eeckhaut T, Leus L, Van Huylenbroeck J. 2011. Mitotic chromosome doubling of plant tissues *in vitro*. Plant Cell, Tissue and Organ Culture **104**:359-373.

Di Pietro R. 2011. New dry grassland associations from the Ausoni-Aurunci mountains (Central Italy)–Syntaxonomical updating and discussion on the higher rank syntaxa. Hacquetia **10**(2).

Diem LT, Phong TH, Tung HT, Khai HD, Anh TTL, Mai NTN, Cuong DM, Luan VQ, Que T, Phuong HTN, Vinh BVT, Nhut DT. 2022. Tetraploid induction through somatic embryogenesis in *Panax vietnamensis* Ha et Grushv. by colchicine treatment. Scientia Horticulturae **303**:111254.

Dodoš T, Janković S, Marin PD, Rajčević N. 2021. Essential oil composition and micromorphological traits of *Satureja montana* L., S. *subspicata* Bartel ex Vis., and *S. kitaibelii* Wierzb. ex Heuff. plant organs. Plants **10**(3):511.

Du J, Wang J, Wang T, Liu L, Iqbal S, Qiao Y. 2021. Identification and chromosome doubling of *Fragaria mandshurica* and *F. nilgerrensis*. Scientia Horticulturae **289**:110507.

Ebadollahi A, Ziaee M, Palla F. 2020. Essential oils extracted from different species of the Lamiaceae plant family as prospective bioagents against several detrimental pests. Molecules **25**(7):1556.

Edris AE. 2007. Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: A Review. Phytherapy Research **21**:308-323.

El-Gazzar A, Watson L. 1970. A taxonomic study of Labiatae and related genera. New Phytologist **69**(2):451-486.

Elgndi MA, Filip S, Pavli´c B, Vladi´c J, Stanojkovi´c T, Žižak Ž, Zekovi´c Z. 2017. Antioxidative and cytotoxic activity of essential oils and extracts of *Satureja montana* L., *Coriandrum sativum* L. and *Ocimum basilicum* L. obtained by supercritical fluid extraction. The Journal of Supercritical Fluids **128**:128-137.

Eng WH, H W.S. 2019. Polyploidization using colchicine in horticultural plants: A review. Scientia Horticulturae **246**:604-617.

Escandón AS, Hagiwara JC, Alderete LM. 2006. A new variety of *Bacopa monnieri* obtained by *in vitro* polyploidization. Electronic Journal of Biotechnology **9**(3):0717-3458.

Estaji A, Hosseini B, Ravandi EG, Dehghan E, Sefidkon F. 2017. The effects of colchicine-induced autotetraploidy on selected characteristics of nuruozak (*Salvia leriifolia*). Cytology and Genetics **51**(1):74-81.

Eurostat. 2019. Eurostat Databaase: Crops Statistics. European Union. Available from https://ec.europa.eu/eurostat/data/database (accessed November 2021).

Fakhrzad F, Jowkar A, Shekafandeh A, Kermani MJ, Moghadam A. 2023. Tetraploidy induction enhances morphological, physiological and biochemical characteristics of wallflower (*Erysimum cheiri* (L.) Crantz). Scientia Horticulturae **308**(27):111596.

Feng W, Chen J, Zheng X, Liu, Q. 2011. Thyme oil to control *Alternaria alternata in vitro* and *in vivo* as fumigant and contact treatments. Food Control **22**(1):78-81.

Fotokian MH, Talei D. 2020. Improving growth indices and productivity of phytochemical compounds in lemon balm (*Melissa officinalis* L.) through induced polyploidy. Biotechnologia **3**:101.

Fox DT, Soltis DE, Soltis PS, Ashman TL, Van de Peer Y. 2020. Polyploidy: A biological force from cells to ecosystems. Trends in Cell Biology **30**:688-694.

Fu L, Zhu Y, Li M, Wang C, Sun H. 2019. Autopolyploid induction via somatic embryogenesis in *Lilium distichum* Nakai and *Lilium cernuum* Komar. Plant Cell, Tissue and Organ Culture **139**:237-248.

Gachkar L, Yadegari D, Rezaei MB, Taghizadeh M, Astaneh SA. Rasooli I. 2007. Chemical and biological characteristics of *Cuminum cyminum* and *Rosmarinus officinalis* essential oils. Food chemistry **102**(3):898-904.

Gallone A, Hunte A, Douglas GC. 2014. Polyploid induction *in vitro* using colchicine and oryzalin on *Hebe* "Oratia Beauty": Production and characterization of the vegetative traits. Scientia Horticulturae **179**:59-66.

Gantait S, Mukherjee E. 2021. Induced autopolyploidy—a promising approach for enhanced biosynthesis of plant secondary metabolites: an insight. Journal of Genetic Engineering and Biotechnology **19**:4.

Gao SL, Zhu DN, Cai ZH, Xu DR. 1996. Autotetraploid plants from colchicine-treated bud culture of *Salvia miltiorrhiza* Bunge. Plant Cell, Tissue and Organ Culture **47**(1):73-77.

Gao SL, Chen BJ, Zhu DN. 2002. *In vitro* production and identification of autotetraploids of *Scutellaria baicalensis*. Plant Cell, Tissue and Organ Culture **70**:289-293.

García-García AL, Grajal-Martín MJ, González-Rodríguez ÁM. 2020. Polyploidization enhances photoprotection in the first stages of *Mangifera indica*. Scientia Horticulturae **264**:109198.

Gärtner H, Schweingruber FH. 2013. Microscopic preparation techniques for plant stem analysis. Remagen, Kessel.

Geoffriau E, Kahane R, Bellamy C, Rancillac M. 1997. Ploidy stability and *in vitro* chromosome doubling in gynogenic clones of onion (*Allium cepa* L.). Plant Science **122**: 201-208.

Ghanbari MA, Jowkar A, Salehi H, Zarei M. 2019. Effects of polyploidization on petal characteristics and optical properties of *Impatiens walleriana* (Hook.). Plant Cell, Tissue and Organ Culture **138**:299-310.

Ghannadi A. 2002. Composition of the essential oil of *Satureja hortensis* L. seeds from Iran. Journal of Essential Oil Research **14**(1):35-36.

Giuliani C, Maleci BL. 2008. Insight into the structure and chemistry of glandular trichomes of Labiatae, with emphasis on subfamily Lamioideae. Plant Systematics and Evolution **276**(3):199-208.

Greplova M, Polzerova H, Domkarova J. 2009. Intra- and inter-specific crosses of *Solanum* materials after mitotic polyploidization *in vitro*. Plant Breeding **128**:651-657.

Güllüce M, Sökmen M, Daferera D, Ağar G, Özkan H, Kartal N, Polissiou M, Sokmen A, Şahin F. 2003. *In vitro* antibacterial, antifungal, and antioxidant activities of the essential oil and methanol extracts of herbal parts and callus cultures of *Satureja hortensis* L. Journal of Agricultural and Food Chemistry **51**(14):3958-3965.

Hannweg K, Steyn W, Bertling I. 2016a. *In vitro*-induced tetraploids of *Plectranthus esculentus* are nematode-tolerant and have enhanced nutritional value. Euphytica **207**(2):343-351.

Hannweg K, Visser G, de Jager K, Bertling I. 2016b. *In vitro*-induced polyploidy and its effect on horticultural characteristics, essential oil composition and bioactivity of *Tetradenia riparia*. South African Journal of Botany **106**:186-191.

Harbard JL, Griffin AR, Foster S, Brooker C, Kha LD, Koutoulis A. 2012. Production of colchicine-induced autotetraploids as a basis for sterility breeding in *Acacia mangium* Willd. Forestry **85**(3):427-436.

Harley RM, Atkins S, Budantsev AL, Cantino PD, Conn BJ, Grayer R, Harley MM, de Kok R, Krestovskaya T, Morales R, Paton AJ, Ryding O, Upson T. 2004. Labiate. Springer, Berlin, Heidelberg.

Harun A, Cai B, Yang Z, Yang Q, Ge X, Li Z. 2021. Asymmetrical effects of autopolyploidization on organ size and gene expression in *Brassica rapa* and *B. oleracea*. Scientia Horticulturae **282**:109991.

Hassanzadeh F, Zakaria RA, Azad NH. 2020. Polyploidy induction in *Salvia officinalis* L. and its effects on some morphological and physiological characteristics. Cytologia **85**(2):157-162.

He J, Huang S, Sun X, Han L, Chang C, Zhang W, Zhong Q. 2019. Carvacrol loaded solid lipid nanoparticles of propylene glycol monopalmitate and glyceryl monostearate:

Preparation, characterization, and synergistic antimicrobial activity. Nanomaterials **9**(8):1162.

Hegarty MJ, Hiscock SJ. 2008. Genomic clues to the evolutionary success of polyploid plants. Current Biology **18**(10):435-444.

Held P. 2005. Performing oxygen radical absorbance capacity assays with synergy TMHT: ORAC antioxidant tests. Application Note. Biotek Instruments, Winooski, USA.

Hernández MD, Sotomayor JA, Hernández Á, Jordán M J. 2016. Essential oils in food preservation, flavor and safety. Academic Press, United States.

Heslop-Harrison P, Schwarzacher T. 2007. Domestication, genomics, and the future for banana. Annals of Botany **100**:1073-1084.

Hu Y, Sun D, Hu H, Zuo X, Xia T, Xie J. 2021. A comparative study on morphological and fruit quality traits of diploid and polyploid carambola (*Averrhoa carambola* L.) genotypes. Scientia Horticulturae **277**:109843.

Huang SS, Kirchoff BK, Liao JP. 2008. The capitate and peltate glandular trichomes of *Lavandula pinnata* L. (Lamiaceae): histochemistry, ultrastructure, and secretion. Journal of Torrey Botanical Society **135**(2):155-167.

Huang H, Gao S, Wang D, Huang P, Li J. 2014. Autotetraploidy induced in nianmaohuangqin (*Radix Scutellariae* viscidulae) with colchicine *in vitro*. Journal of Traditional Chinese Medicine **34**:199-205.

Iannicelli J, Elechosa MA, Juarez MA, Martinez A, Bugallo V, Bandoni AL, Escandon AS, van Baren CM. 2016. Effect of polyploidization in the production of essential oils in *Lippia integrifolia*. Industrial Crops and Products **81**:20-29.

Iannicelli J, Guariniello J, Tossi V, Regalado J, Di Ciaccio L, Van Baren C, Álvarez S, Escandon A. 2020. The "polyploid effect" in the breeding of aromatic and medicinal species. Scientia Horticulturae **260**:108854.

Iapichino G, Arnone C, Bertolino M, Roxas UA. 2006. Propagation of three *Thymus* species by stem cuttings. Acta Horticulturae **723**:411-414.

Innan H, Kondrashov F. 2010. The evolution of gene duplications: classifying and distinguishing between models. Nature Reviews Genetics **11**:97.

Isman MB. 2006. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annual Review of Entomology **51**:45-66.

Isman MB, Seffrin R. 2018. Essential oils as green pesticides for plant protection in horticulture. Sustainable Horticulture. Apple Academic Press, Florida, United States.

Jaafari A, Mouse HA, Rakib EM. 2007. Chemical composition and antitumor activity of different wild varieties of Moroccan thyme. Revista Brasileira de Farmacognosia **17**(4):477-491.

Jafari F, Ghavidel F, Zarshenas MM. 2016. A critical overview on the pharmacological and clinical aspects of popular *Satureja species*. Journal of Acupuncture and Meridian Studies **9**(3):118-127.

Jarić S, Mačukanović-Jocićb M, Djurdjević L, Mitrović M, Kostić O, Karadžić B, Pavlović P. 2015. An ethnobotanical survey of traditionally used plants on Suva planina mountain (south eastern-Serbia). Journal of Ethnopharmacology **175**:93-108.

Javanmardi Z, Saba MK, Nourbakhsh H, Amini J. 2023. Efficiency of nanoemulsion of essential oils to control *Botrytis cinerea* on strawberry surface and prolong fruit shelf life. International Journal of Food Microbiology **384**:109979.

Jiang L, Wang X, Geng Z, Wang Y, Liu L, Wang T, Yu J, Li M, Zhao X. 2022. Autopolyploidy enhances agronomic traits and active ingredients in 'Huaibai', a top-grade medicinal chrysanthemum. Plant Cell, Tissue and Organ Culture **151**:433-442.

Jokari S, Shekafandeh A, Jowkar A. 2022. *In vitro* tetraploidy induction in Mexican lime and sour orange and evaluation of their morphological and physiological characteristics. Plant Cell, Tissue and Organ Culture **150**:651-668.

Julião SA, Ribeiro CV, Lopes JML, de Matos EM, Reis AC, Peixoto PHP, Machado MA, Azevedo ALS, Grazul RM, de Campos JMS, Viccini LF. 2020. Induction of synthetic polyploids and assessment of genomic stability in *Lippia alba*. Frontiers in Plant Science **11**:292.

Kamble P, Giri, SP, Mane RS, Tiwana A. 2015. Estimation of chlorophyll content in young and adult leaves of some selected plants. Universal Journal of Environmental Research and Technology **5**(6):306-310.

Kremer D, Kosir IJ, Koncic MZ, Cerenak A, Potocnik T, Srecec S, Kosalec I. 2015. Antimicrobial and antioxidant properties of *Satureja montana* L. and *S. subspicata* Vis.(Lamiaceae). Current Drug Targets **16**(14):1623-1633.

Khalid AH, Jin HJ. 2017. Chemical composition of neem and lavender essential oils and their antifungal activity against pathogenic fungi causing ginseng root rot. African Journal of Biotechnology **16**(52):2349-2354.

Khan T, Khan MA, Mashwani ZR, Ullah N, Nadhman A. 2021. Therapeutic potential of medicinal plants against COVID-19: The role of antiviral medicinal metabolites. Biocatalysis and Agricultural Biotechnology **31**:101890.

Khezri M, Asghari-Zakaria R, Zare N, Johari-Ahar M. 2022. Tetraploidy induction increases galegine content in *Galega officinalis* L. Journal of Applied Research on Medicinal and Aromatic Plants **4**:100366.

Khoury M, Stien D, Eparvier V, Ouaini N, El Beyrouthy M. 2016. Report on the medicinal use of eleven Lamiaceae species in Lebanon and rationalization of their antimicrobial potential by examination of the chemical composition and antimicrobial activity of their essential oils. Evidence-Based Complementary and Alternative Medicine 17.

Klíma M, Vyvadilová M, Kučera V. 2008. Chromosome doubling effects of selected antimitotic agents in *Brassica napus* microspore culture. Czech Journal of Genetics and Plant Breeding **44**(1):30-36.

Kokkini S, Karousou R, Hanlidou E. 2003. Encyclopedia of Food Sciences and Nutrition. Academic Press, United States.

Kokoska L, Kloucek P, Leuner O, Novy P. 2019. Plant-derived products as antibacterial and antifungal agents in human health care. Current Medicinal Chemistry **26**(29):5501-5541.

Kolodziejek J, Michlewscka, S. 2015. Effect of soil moisture on mor-pho-anatomical leaf traits of *Ranunculus acris* (Ranunculaceae). Polish Journal of Ecology **63**:400-413.

Kondrashov FA. 2012. Gene duplication as a mechanism of genomic adaptation to a changing environment. Proceedings of the Royal Society B: Biological Sciences **279**:5048-5057.

Krause ST, Liao P, Crocoll C, Boachon B, Forster C, Leidecker F, Wiese N, Zhao D, Wood JC, Buell CR, Gershenzon J, Dudareva N, Degenhardt J. 2021. The biosynthesis of thymol, carvacrol, and thymohydroquinone in Lamiaceae proceeds via cytochrome P450s and a short-chain dehydrogenase. Proceedings of the National Academy of Sciences **118**(52):e2110092118.

Kumar K, Rao IU. 2012. Morphophysiological problems in acclimatization of micropropagated plants in - *ex vitro* conditions - a review. Journal of Ornamental and Horticultural Plants **2**(4):271-283.

Kurkcuoglu M, Tumen G, Baser KHC. 2001. Essential oil constituents of *Satureja boissieri* from Turkey. Chemistry of Natural Compounds **37**(4):329-331.

Lan MO, Chen JH, Chen F, Xu QW, Tong ZK, Huang HH, Dong RH, Lou XZ, Lin EP. 2020. Induction and characterization of polyploids from seeds of *Rhododendron fortunei* Lindl. Journal of Integrative Agriculture **19**(8):2016-2026.

Lattier JD, Touchell DH, Ranney TG, Smith JC. 2013. Micropropagation and polyploid induction of *Acer platanoides* crimson sentry. Journal of Environmental Horticulture **31**(4):246-252.

Lemos MF, Lemos MF, Pacheco HP, Guimarães AC, Fronza M, Endringer DC, Scherer R. 2017. Seasonal variation affects the composition and antibacterial and antioxidant activities of *Thymus vulgaris*. Industrial Crops and Products **95**:543-548.

Leonti M, Casu L, Sanna F, Bonsignore L. 2009. A comparison of medicinal plant use in Sardinia and Sicily–De Materia Medica revisited? Journal of Ethnopharmacology **121**:255-267.

Leus L, Van Laere K, Dewitte A, Van Huylenbroeck J. 2009. Flow cytometry for plant breeding. Acta Horticulturae **836**:221-226.

Li XW, Zhang ZJ, Hafeez M, Huang J, Zhang JM, Wang LK, Lu YB. 2021. *Rosmarinus officinialis* L. (Lamiales: Lamiaceae), a promising repellent plant for thrips management. Journal of Economic Entomology **114**(1):131-141.

Liu B, Chen B, Zhang J, Wang P, Feng G. 2017. The environmental fate of thymol, a novel botanical pesticide, in tropical agricultural soil and water. Toxicological and Environmental Chemistry **99**(2):223-232.

Liu C, Liu T, Ohlson EW, Wang L, Wu D, Guo Q, Timko MP, Liang G. 2019. Loquat (*Eriobotrya japonica* (Thumb.) circadian clock gene cloning and heterosis studies of artificial triploid loquat. Scientia Horticulturae **246**:328-337.

Liu J, Li J, Fu C. 2021a. Comparative physiology and transcriptome analysis reveals the regulatory mechanism of genome duplication enhancing cold resistance in *Fragaria nilgerrensis*. Environmental and Experimental Botany **188**:104509.

Liu R, Gao C, Jin J, Ma H, Zhang Y, Qi B, Xu J. 2022. Induction and identification of tetraploids of pear plants (*Pyrus bretschneideri* and *Pyrus betulaefolia*). Scientia Horticulturae **304**: 111322.

Lugasi A, Hovari J, Hagymasi K, Jakoczi I, Blazovics A. 2006. Antioxidant properties of a mixture of Lamiaceae plants intended to use as a food additive. Acta Alimentaria **35**(1):85-97.

López-Cobo A, Gómez-Caravaca AM, Švarc-Gajić J, Segura-Carretero A, Fernández-Gutiérrez A. 2015. Determination of phenolic compounds and antioxidant activity of a Mediterranean plant: The case of *Satureja montana* subsp. *kitaibelii*. Journal of Functional Foods **18**:1167-1178.

Lukošiūtė S, Šernaitė L, Morkeliūnė A, Rasiukevičiūtė N, Valiuškaitė A. 2020. The effect of Lamiaceae plants essential oils on fungal plant pathogens *in vitro*. Agronomy Research **18**(4):2761-2769.

Lumpert M, Kreft S. 2017. Folk use of medicinal plants in Karst and Gorjanci, Slovenia. Journal of Ethnobiology and Ethnomedicine **13**(1):1-34.

Machado RM, de Oliveira FA, Alves FM, de Souza AP, Forni-Martins ER. 2020. Population genetics of polyploid complex *Psidium cattleyanum* Sabine (Myrtaceae): Preliminary analyses based on new species-specific microsatellite loci and extension to other species of the genus. Biochemistry **59**:219-234.

Makova KD. Li WH. 2003. Divergence in the spatial pattern of gene expression between human duplicate genes. Genome Research **13**:1638-1645.

Malmanche N, Maia A, Sunkel CE. 2006. The spindle assembly checkpoint: Preventing chromosome mis-segregation during mitosis and meiosis. FEBS Letters **580**(12): 2888-2895.

Mamgain A, Roychowdhury R, Tah J. 2013. Alternaria pathogenicity and its strategic controls. Research Journal of Biology **1**:1-9.

Mandal S, DebMandal M. 2016. Essential oils in food preservation, flavor and safety. Academic Press, United States.

Manzoor A, Ahmad T, Bashir M, Baig M, Quresh A, Shah M, Hafiz I. 2018. Induction and identification of colchicine induced polyploidy in *Gladiolus grandiflorus* 'White Prosperity'. Folia Horticulturae **30**(2):307-319.

Manzoor A, Ahmad T, Bashir MA, Hafiz IA, Silvestri C. 2019. Studies on colchicine induced chromosome doubling for enhancement of quality traits in ornamental plants. Plants **8**(7):194.

Marchese A, Orhan IE, Daglia M, Barbieri R, Lorenzo AD, Nabavi SF, Gortzi O, Izadi M, Nabavi S.M. 2016. Antibacterial and antifungal activities of thymol: A brief review of the literature. Food Chemistry **210**:402-414.

Marfil CF, Duarte PF, Masuelli RW. 2018. Phenotypic and epigenetic variation induced in newly synthesized allopolyploids and autopolyploids of potato. Scientia Horticulturae **234**:101-109.

Marin M, Ascensão L, Marin PD. 2012. Microscopic investigation of trichomes of wild-growing *Satureja montana* L. Archives of Biological Sciences **64**(4):1599-1605.

Matthias A, Laisne N. 2017. Medicinal plants: production, cultivation and uses. Nova Science Publishers Inc. Hauppauge, New York.

Matkowski A, Piotrowska M. 2006. Antioxidant and free radical scavenging activities of some medicinal plants from the Lamiaceae. Fitoterapia **77**(5):346-353.

Matusinsky P, Zouhar M, Pavela R, Novy P. 2015. Antifungal effect of five essential oils against important pathogenic fungi of cereals. Industrial Crops and Products **67**:208-215.

Mei B, Xie H, Xing H, Kong D, Pan X, Li Y, Wu H. 2020. Changes of Phenolic Acids and Antioxidant Activities in Diploid and Tetraploid *Echinacea purpurea* at Different Growth Stages. Revista Brasileira de Farmacognosia **30**:510-518.

Mchedlishvili D, Kuchukashvili Z, Tabatadze T, Davitaia G. 2005. Influence of flavonoids isolated from *Satureja hortensis* L. on hypercholesterolemic rabbits. Indian Journal of Pharmacology **37**(4):259.

Mihajilov-Krstev T, Radnović D, Kitić D, Jovanović V, Mitić V, Stojanović-Radić Z, Zlatković B. 2014. Chemical composition, antimicrobial, antioxidative and anticholinesterase activity of *Satureja montana* L. ssp *montana* essential oil. Open Life Sciences **9**(7):668-677.

Mirjana S, Nada B. 2004. Chemical composition and antimicrobial variability of *Satureja montana* L. essential oils produced during ontogenesis. Journal of Essential Oil Research **16**(4):387-391.

Mirjani L, Salimi A, Matinizadeh M, Razavi K, Shahbazi M. Effective factors on micropropagation of medicinal plant of *Satureja khuzistanica*. Iranian Journal of Rangelands and Forests Plant Breeding and Genetic Research **26**(1).

Mitrofanova IV, Zilbervarg IR, Yemets AI, Mitrofanova OV, Blume YB. 2003. The effect of dinitroaniline and phosphorothioamidate herbicides on polyploidisation *in vitro* of nepeta plants. Cell Biology International **27**:229-231.

Mo L, Chen J, Lou X, Xu Q, Dong R, Tong Z, Huang H, Lin E. 2020. Colchicine-induced polyploidy in *Rhododendron fortunei* Lindl. Plants **9**(4):424.

Modenesi P, Serrato-Valenti G, Bruni A. 1984. Development and secretion of clubbed trichomes in *Thymus vulgaris* L. Flora **175**(3):211-219.

Moetamedipoor SA, Jowkar A, Saharkhiz MJ, Hassani HS. 2022. Hexaploidy induction improves morphological, physiological and phytochemical characteristics of mojito mint (*Mentha* × *villosa*). Scientia Horticulturae **295**:110810.

Momtaz S, Abdollahi M. 2008. A systematic review of the biological activities of *Satureja* L. species. Pharmacologyonline **2**:34-54.

Mosa KA, El-Naggar M, Ramamoorthy K, Alawadhi H, Elnaggar A, Wartanian S, Ibrahim E, Hani H. 2018. Copper nanoparticles induced genotoxicty, oxidative stress, and changes

in superoxide dismutase (SOD) gene expression in cucumber (*Cucumis sativus*) plants. Frontiers in Plant Science **9**:872.

Mozafari AA, Vafaee Y, Karami E. 2015. *In vitro* propagation and conservation of *Satureja avromanica* Maroofi—an indigenous threatened medicinal plant of Iran. Physiology and Molecular Biology of Plants **21**(3):433-439.

Murashige T, Nakano R. 1966. Tissue culture as a potential tool in obtaining polyploid plants. Journal of Heredity **57**:115-118.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum **15**:473-497.

Nabavi SM, Marchese A, Izadi M, Curti V, Daglia M, Nabavi SF. 2015. Plants belonging to the genus *Thymus* as antibacterial agents: From farm to pharmacy. Food Chemistry **173** :339-347.

Nakatsu T, Lupo Jr AT, Chinn Jr JW, Kang RK. 2000. Biological activity of essential oils and their constituents. Studies in Natural Products Chemistry **21**:571-631.

Napoli EM, Curcuruto G, Ruberto G. 2010. Screening of the essential oil composition of wild Sicilian Thyme. Biochemical Systematics and Ecology **38**:816-822.

Nassar NMA, Graciano Ribeiro D, Fernandes SDC, Araujo PC. 2008. Anatomical alterations due to polyploidy in cassava, *Manihot esculenta* Crantz. Genetics and Molecular Research **7**:276-283.

Navarro-Rocha J, Andrés MF, Díaz CE, Burillo J, González-Coloma A. 2020. Composition and biocidal properties of essential oil from pre-domesticated Spanish *Satureja montana*. Industrial Crops and Products **145**:111958.

Neocleous D, Ntatsi G. 2018. Seasonal variations of antioxidants and other agronomic features in soilless production of selected fresh aromatic herbs. Scientia Horticulturae **234**:290-299.

Niazi M, Yari F, Shakarami A. 2019. A review of medicinal herbs in the lamiaceae family used to treat arterial hypertension. Entomology and Applied Science Letters **6**(1):22-7.

Niazian M, Nalousi AM. 2020. Artificial polyploidy induction for improvement of ornamental and medicinal plants. Plant Cell, Tissue and Organ Cultures **142**:447-469.

Nicola S, Fontana E, Hoeberechts J. 2004. Cultural techniques to optimize the thyme (*Thymus vulgaris*) propagation. Acta Horticulturae **631**:187-192.

Nieto G. 2017. Biological activities of three essential oils of the Lamiaceae family. Medicines **4**(3):63.

Noori SAS, Norouzi M, Karimzadeh G, Shirkool K, Niazian M. 2017. Effect of colchicine-induced polyploidy on morphological characteristics and essential oil composition of ajowan (*Trachyspermum ammi* L.). Plant Cell, Tissue and Organ Culture **130**:543-551.

Novák J, Skalický M. 2017. Botanika: cytologie, histologie, organologie a systematika. Powerprint. Prague.

Novák J, Blüthner WD. 2020. Medicinal, aromatic and stimulant plants. Springer. Switzerland.

Okach DO, Nyunja ARO, Opande G. 2013. Phytochemical screening of some wild plants from Lamiaceae and their role in traditional medicine in Uriri District-Kenya. International Journal of Herbal Medicine **1**(5):135-143.

Okigbo RN, Anuagasi CL, Amadi JE. 2009. Advances in selected medicinal and aromatic plants indigenous to Africa. Journal of Medicinal Plants Research **3**(2):86-95.

Oliveira-Pinto PR, Mariz-Ponte N, Torres A, Tavares F, Fernandes-Ferreira M, Sousa RM, Santos C. 2022. *Satureja montana* L. essential oil, montmorillonite and nanoformulation reduce *Xanthomonas euvesicatoria* infection, modulating redox and hormonal pathways of tomato plants. Scientia Horticulturae **295**:110861.

Omidbaigi R, Mirzaee M, Hassani ME, Moghadam MS. 2010a. Induction and identification of polyploidy in basil (*Ocimum basilicum* L.) medicinal plant by colchicine treatment. International Journal of Plant Production **4**(2):1735-8043.

Omidbaigi R, Yavari S, Hassani ME, Yavari S. 2010b. I nduction of autotetraploidy in dragonhead (*Dracephalum moldavica* L.) by colchicine treatment. Journal Fruit and Ornamental Plant Research **18**:23-35.

Ou B, Hampsch-Woodill M, Prior RL. 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. Journal of Agricultural and Food Chemistry **49**:4619-4626.

Otto SP. 2007. The evolutionary consequences of polyploidy. Cell **131**:452-462.

Özcan M, Chalchat J. 2004. Aroma profile of *Thymus vulgaris* L. growing wild in Turkey. Bulgarian Journal of Plant Physiology **30**:68-73.

Özkan M. 2008. Glandular and eglandular hairs of *Salvia recognita* Fisch & Mey (Lamiaceae) in Turkey. Journal of Botany **37**(1):93-95.

Pandey AK, Kumar P, Singh P, Tripathi NN, Bajpai VK. 2017. Essential oils: Sources of antimicrobials and food preservatives. Frontiers in Microbiology **7**:1-14.

Parasath D, Nair RR, Babu PA. 2022. Effect of colchicine induced tetraploids of ginger (*Zingiber officinale* Roscoe) on cytology, rhizome morphology, and essential oil content. Journal of Applied Research on Medicinal and Aromatic Plants **31**:100422.

Park JH, Jeon YJ, Lee CH, Chung N, Lee HS. 2017. Insecticidal toxicities of carvacrol and thymol derived from *Thymus vulgaris* Lin. against *Pochazia shantungensis* Chou & Lu., newly recorded pest. Scientific Reports **7**:1-7.

Pavela R. 2007. Possibilities of botanical insecticide exploitation in plant protection. Pest Technology **1**(1):47-52.

Pavela R, Vrchotová N, Tříska J. 2009. Mosquitocidal activities of thyme oils (*Thymus vulgaris* L.) against *Culex quinquefasciatus* (Diptera: Culicidae). Parasitology Research **105**:1365.

Pavela R. 2011. Insecticidal and repellent activity of selected essential oils against of the pollen beetle, *Meligethes aeneus* (Fabricius) adults. Industrial Crops and Products **34**(1):888-892.

Pavela R, Benelli G. 2016. Essential oils as ecofriendly biopesticides? Challenges and constraints. Trends in Plant Science **21**:1000-1007.

Pavela R, Zabka M, Vrchotova N, Triska J. 2018. Effect of foliar nutrition on the essential oil yield of Thyme (*Thymus vulgaris* L.). Industrial Crops and Products **112**:762-765.

Pavlović MO, Lunić T, Graovac S, Mandić M, Repac J, Gašić U, Nedeljković BB, Božić B. 2022. Extracts of selected Lamiaceae species as promising antidiabetics: Chemical profiling, *in vitro* and *in silico* approach combined with dynamical modeling. Industrial Crops and Products **186**:115200.

Pei Y, Yao N, He L, Deng D, Li W, Zhang W. 2019. Comparative study of the morphological, physiological and molecular characteristics between diploid and tetraploid radish (*Raphunas sativus* L.). Scientia Horticulturae **257**:108739.

Peterson JJ, Dwyer JT, Jacques PF, McCullough ML. 2012. Associations between flavonoids and cardiovascular disease incidence or mortality in European and US populations. Nutrition Reviews **70**(9):491-508.

Pinto L, Cefola M, Bonifacio MA, Cometa S, Bocchino C, Pace B, De Giglio E, Palumbo M, Sada A, Logrieco AF, Baruzzi F. 2021. Effect of red thyme oil (*Thymus vulgaris* L.) vapours on fungal decay, quality parameters and shelf-life of oranges during cold storage. Food Chemistry **336**:127590.

Pospíšilová J, Synková H, Haisel D, Semorádová Š. 2007. Acclimation of plantlets to *ex vitro* conditions: Effects of air humidity, irradiance, CO2 concentration and Abscisic acid (a review). Acta Horticulturae **748**:29-38.

R Core Team. 2021. R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria. Available from https://www.R-project.org/ (accessed June 2021).

Rahimzadeh S, Sohrabi Y, Heidari G, Pirzad A, Golezani KG. 2016. Effect of bio-fertilizers on the essential oil yield and components isolated from *Dracocephalum moldavica* L. using nanoscale injection method. Journal of Essential Oil Bearing Plants **19**(3):529-541.

Raja R. 2012. Medicinally potential plants of Labiatae (Lamiaceae) Family: An overview. Research Journal of Medicinal Plants **6**:203-213.

Ramanna MS, Jacobsen E. 2003. Relevance of sexual polyploidization for crop improvement – A review. Euphytica **133**:3-18.

Ranney TG. 2006. Polyploidy: From evolution to new plant development. Combined Proceedings International Plant Propagators' Society **56**:137-142.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine **26**:1231-1237.

Reang SP, Mishra JP, Prasad R. 2020. *In vitro* antifungal activities of five plant essential oils against *Botrytis cinerea* causing gray mold of orange. Journal of Pharmacognosy and Phytochemistry **9**(3):1046-1048.

Redpath LE, Aryal R, Lynch N, Spencer JA, Hulse-Kemp AM, Ballington JR, Green J, Bassil N, Hummer K, Ranney T, Ashrafi H. 2022. Nuclear DNA contents and ploidy levels of North American *Vaccinium* species and interspecific hybrids. Scientia Horticulturae **297**:110995.

Redzic S. 2010. Wild medicinal plants and their usage in traditional human therapy (Southern Bosnia and Herzegovina, W. Balkan). Journal of Medicinal Plants Research **4**(11):1003-1027.

Rotolo V, Barresi G, Di Carlo E, Giordano A, Lombardo G, Crimi E, Costa E, Bruno M, Palla F. 2016. Plant extracts as green potential strategies to control the biodeterioration of cultural heritage. International Journal Conservation Science **7**(2):839-846.

Rotolo V, De Caro ML, Giordano A, Palla F. 2018. Solunto archaeological park in Sicily: Life under mosaic tesserae. Flora Mediterranea **28**:233-245.

Rowland LS, Smith HK, Taylor G. 2018. The potential to improve culinary herb crop quality with deficit irrigation. Scientia Horticulturae **242**:44-50.

Rice A, Glick L, Abadi S, Einhorn M, Kopelman NM, Minkov AS, Mayzel J, Chay O. Mayrose I. 2014. The chromosome counts database (CCDB) – a community resource of plant chromosome numbers. New Phytologist **206**(1):19-26.

Rivera D, Verde A, Fajardo J, Obón C, Consuegra V, García-Botía J, Ríos S, Alcaraz F, Valdés A, del Moral A, Laguna E. 2019. Ethnopharmacology in the upper Guadiana River area (Castile-La Mancha, Spain). Journal of Ethnopharmacology **241**:111968.

Sá RCS, Andrade LN, De Sousa DP. 2015. Sesquiterpenes from Essential Oils and Anti-Inflammatory Activity. Natural Product Communications **10**(10):1767-1774.

Saad MM, El-Deeb DA, Abdelgaleil SA. 2019. Insecticidal potential and repellent and biochemical effects of phenylpropenes and monoterpenes on the red flour beetle, *Tribolium castaneum* Herbst. Environmental Research **26**(7):6801-6810.

Sabooni N, Gharaghani A, Jowkar A, Eshghi S. 2022. Successful polyploidy induction and detection in blackberry species by using an *in vitro* protocol. Scientia Horticulturae **295**:110850.

Salehi B, Mishra AP, Shukla I, Sharifi-Rad M, Contreras, MM, Segura-Carretero A, Fathi H, Nasrabadi NN, Kobarfard F, Sharifi-Rad J. 2018. Thymol, thyme, and other plant sources: Health and potential uses. Phytherapy Research **32**:1688-1706.

Salma U, Kundu S, Mandal N. 2017. Artificial polyploidy in medicinal plants: Advancement in the last two decades and impending prospects. Journal of Crop Science and Biotechnology **20**:9-19.

Santos JD, Coelho E, Silva R, Passos CP, Teixeira P, Henriques I, Coimbra MA. 2019. Chemical composition and antimicrobial activity of *Satureja montana* byproducts essential oils. Industrial Crops and Products **137**:541-548.

Sarac N, Uğur, A. 2007. Antimicrobial activities and usage in folkloric medicine of some Lamiaceae species growing in Mugla, Turkey. EurAsian Journal of BioSciences **4**:28-37.

Sarropoulou V, Maloupa E. 2019. *In vitro* propagation of *Satureja thymbra* L. (Lamiaceae): A valuable aromaticmedicinal native plant of the Mediterranean region. GSC biological and pharmaceutical sciences **9**(2):9-20.

Sattler MC, Carvalho CR, Clarindo WR. 2016. The polyploidy and its key role in plant breeding. Planta **243**:281-296.

Sediva J, Mrazkova M, Zahumenicka P, Cusimamani EF, Zahradnik D. 2019. Identification of *Phytophthora* tolerance in the *Anemone sylvestris* tetraploid. Scientia Horticulturae **256**:108579.

Sellamuthu PS, Sivakumar D, Soundy P. 2013. Antifungal activity and chemical composition of thyme, peppermint and citronella oils in vapor phase against avocado and peach postharvest pathogens. Journal of Food Safety **33**:86-93.

Sertel S, Eichhorn T, Plinkert P. 2011. Cytotoxicity of *Thymus vulgaris* essential oil towards human oral cavity squamous cell carcinoma. Anticancer Research **31**:81-87.

Shaikh N, Verma RC, Khah MA, Filimban FZ, Albaz AA, Alam Q. 2021. Morphological and chromosomal characterization of diploid and colchicine induced autotetraploid plants of *Commelina benghalensis* L. (Commelinaceae). Cytologia **86**(2):167-173.

Shao J, Chen C, Deng X. 2003. *In vitro* induction of tetraploid in pomegranate (*Punica granatum*). Plant Cell, Tissue and Organ Culture **75**:241-246.

Shariat A, Karimzadeh G, Assareh MH. 2013. Karyology of Iranian Endemic Satureja (Lamiaceae) Species. Cytologia **78**(3):305-312.

Sharma OP, Bhat TK. 2009. DPPH antioxidant assay revisited. Food Chemistry **113**:1202-1205.

Shi L, Gao S, Lei T, Duan Y, Yang L, Li J, Zhao Z, Ling Q. 2022. An integrated strategy for polyploidization of *Cerastigma willmottianum* Stapf based on tissue culture and chemical mutagenesis and the carbon dioxide fixation ability of tetraploids. Plant Cell, Tissue and Organ Culture **149**:767-782.

Shmeit YH, Fernandez E, Novy P, Kloucek P, Orosz M, Kokoska L. 2020. Autopolyploidy effect on morphological variation and essential oil content in *Thymus vulgaris* L Scientia Horticulturae **263**:109095.

Silva AS, Tewari D, Sureda A, Suntar I, Belwal T, Battino M, Nabavi SM, Nabavi SF. 2021. The evidence of health benefits and food applications of *Thymus vulgaris* L. Trends in Food Science and Technology **117**:218-227.

Singleton VL, Orthofer R, Lamuela-Raventos RM. 1998. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods of Enzymology **299**:152-178.

Skendi A, Katsantonis DN, Chatzopoulou P, Irakli M, Papageorgiou M. 2020. Antifungal activity of aromatic plants of the lamiaceae family in bread. Foods **9**(11):1642.

Slavkovska V, Jancic R, Bojovic S, Milosavljevic S, Djokovic D. 2001. Variability of essential oils of *Satureja montana* L. and *Satureja kitaibelii* Wierzb. ex Heuff. from the central part of the Balkan peninsula. Phytochemistry **57**(1):71-76.

Sliwinska E. 2018. Flow cytometry – a modern method for exploring genome size and nuclear DNA synthesis in horticultural and medicinal plant species. Folia Horticulturae **30**(1):103-128.

Soltis DE, Visger CJ, Marchant DB, Soltis PS. 2016. Polyploidy: Pitfalls and paths to a paradigm. American Journal of Botany **103**(7):1146-66.

Sotelo JP, Oddino C, Giordano GF, Carezzano ME, Oliva MM. 2021. Effect of *Thymus vulgaris* essential oil on soybeans seeds infected with *Pseudomonas syringae*. Physiological and Molecular Plant Pathology **116**:101735.

Souto-Maior FN, Fonsêca DVD, Salgado PRR, Monte LDO, de Sousa DP, de Almeida RN. 2017. Antinociceptive and anticonvulsant effects of the monoterpene linalool oxide. Pharmaceutical Biology **55**(1):63-67.

Sow LC, Tirtawinata F, Yang H, Shao Q, Wang S. 2017. Carvacrol nanoemulsion combined with acid electrolysed water to inactivate bacteria, yeast *in vitro* and native microflora on shredded cabbages. Food Control **76**:88-95.

Sun M, Zhang Y, Zhu L, Liu N, Bai H, Sun G, Zhang J, Shi L. 2022. Chromosome-level assembly and analysis of the *Thymus* genome provide insights into glandular secretory trichome formation and monoterpenoid biosynthesis in thyme. Plant Communications **3**(6):100413.

Stahl-Biskup E, Sáez F. 2002. Thyme: the genus Thymus. Taylor & Francis, London.

Stahl-Biskup E, Venskutonis RP. 2012. Thyme. In Handbook of herbs and spices. Woodhead Publishing **1**:499-525.

Storchova Z, 2014. Ploidy changes and genome stability in yeast. Yeast **31**(11):421-430.

Šojić B. Pavlić B, Tomović V, Ikonić P, Zeković Z, Kocić-Tanackov S, Đurović S, Škaljac S, Jokanović M, Ivić M. 2019. Essential oil versus supercritical fluid extracts of winter savory (*Satureja montana* L.)–Assessment of the oxidative, microbiological and sensory quality of fresh pork sausages. Food Chemistry **287**:280-286.

Taïbi K, Abderrahim LA, Ferhat K, Betta S, Taïbi F, Bourrada F, Boussaid M. 2020. Ethnopharmacological study of natural products used for traditional cancer therapy in Algeria. Saudi Pharmaceutical Journal **28**:1451-1465.

Talei D, Fotokian MH. 2020. Improving growth indices and productivity of phytochemical compounds in lemon balm (*Melissa officinalis* L.) through induced polyploidy. BioTechnologia **101**(3):215-226.

Tan FQ, Tu H, Liang WJ, Long JM, Wu XM, Zhang HY, Guo WW. 2015. Comparative metabolic and transcriptional analysis of a doubled diploid and its diploid citrus

rootstock (*C. junos* cv. Ziyang xiangcheng) suggests its potential value for stress resistance improvement. BMC Plant Biology **15**:89.

Tan FQ, Tu H, Wang R, Wu XM, Xie KD, Chen JJ, Zhang HY, Xu J, Guo WW. 2017. Metabolic adaptation following genome doubling in citrus doubled diploids revealed by non-targeted metabolomics. Metabolomics **13**:143.

Tan FQ, Zhang M, Xie KD, Fan YJ, Song X, Wang R, Wu XM, Zhang HY, Guo WW. 2019. Polyploidy remodels fruit metabolism by modifying carbon source utilization and metabolic flux in Ponkan mandarin. Plant Science **289**:110276.

Tavan M, Mirjalili MH, Karimzadeh G. 2015. *In vitro* polyploidy induction: Changes in morphological, anatomical and phytochemical characteristics of *Thymus persicus* (Lamiaceae). Plant Cell, Tissue and Organ Culture **122**:573-583.

Tavan M, Sarikhani H, Mirjalili MH, Rigano MM, Azizi A. 2021. Triterpenic and phenolic acids production changed in *Salvia officinalis* via *in vitro* and *ex vitro* polyploidization: A consequence of altered genes expression. Phytochemistry **189**:112803.

Tavan M, Azizi A, Sarikhani H, Mirjalili MH, Rigano MM. 2022. Induced polyploidy and broad variation in phytochemical traits and altered gene expression in *Salvia multicaulis.* Scientia Horticulturae **291**:110592.

Tambong JJ, Sapta VT, Garton S. 1998. *In vitro* induction of tetraploids in colchicine-treated cocyam plantlets. Euphytica **104**:191-197.

Teshome S, Soromessa T, Feyissa T. 2017. *In vitro* propagation of a threatened medicinal plant *Satureja abyssinica* through nodal explants – Antimicrobial and antifungal herb. International Journal of BioSciences and Technology **10**(3):20-25.

Theis N, Lerdau M. 2003. The evolution of function in plant secondary metabolites. International Journal of Plant Sciences **164**(3):93-102.

Tholl D. 2006. Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. Current Opinion in Plant Biology **9**(3):297-304.

Tomaselli V, Silletti G, Forte L. 2021. A new association of *Satureja montana* L. subsp. *montana* dominated garrigues in Puglia (SE Italy). Plant Sociology **58**:1.

Topcu G, Kusman T. 2014. Lamiaceae family plants as a potential anticholinesterase source in the treatment of Alzheimer's disease. Bezmialem Science **2**(1):1-25.

Trindade H, Pedro LG, Figueiredo AC, Barroso JG. 2018. Chemotypes and terpene synthase genes in *Thymus* genus: State of the art. Industrial Crops and Products **124**:530-547.

Tsuro M, Kondo N, Noda M, Ota K, Nakao Y, Asada S. 2016. *In vitro* induction of autotetraploid of Roman chamomile (*Chamaemelum nobile* L.) by colchicine treatment

and essential oil productivity of its capitulum. *In Vitro* Cellular and Developmental Biology - Plant **52**:479-483.

Urwin NAR, Horsnell J, Moon T. 2007. Generation and characterisation of colchicine-induced autotetraploid *Lavandula angustifolia.* Euphytica **156**:257-266.

Urwin NAR. 2014. Generation and characterisation of colchicine-induced polyploid *Lavandula* × *intermedia*. Euphytica **197**(3):331-339.

Van de Peer Y, Mizrachi E. Marchal K. 2017. The evolutionary significance of polyploidy. Nature Reviews Genetics **18**:411.

Van den Top GG, Reynolds JD, Prins HH, Mattsson J, Green DJ, Ydenberg RC. 2018. From salmon to salmonberry: the effects of salmon-derived nutrients on the stomatal density of leaves of the nitriphilic shrub *Rubus spectabilis*. Functional Ecology **32**(11):2625-2633.

Vasilijević B, Mitić-Ćulafić D, Djekic I, Marković T, Knežević-Vukčević J, Tomasevic I, Velebit B, Nikolić B. 2019. Antibacterial effect of *Juniperus communis* and *Satureja montana* essential oils against *Listeria monocytogenes in vitro* and in wine marinated beef. Food control **100**:247-256.

Viehmannová I, Cusimamani EF, Bechyne M, Vyvadilová M, Greplová M. 2009. *In vitro* induction of polyploidy in yacon (*Smallanthus sonchifolius*). Plant Cell, Tissue and Organ Culture **97**:21-25.

Verdeguer M, Roselló J, Castell V, Llorens JA, Santamarina MP. 2020. Cherry tomato and persimmon kaki conservation with a natural and biodegradable film. Current Research in Food Science **2**:33-40.

von Arx G, Crivellaro A, Prendin AL, Čufar K, Carrer M. 2016. Quantitative wood anatomy—Practical guidelines. Frontiers in Plant Science **7**:781.

Wang L, Luo Z, Wang L, Deng W, Wei H, Liu P, Liu M. 2019. Morphological, cytological and nutritional changes of autotetraploid compared to its diploid counterpart in Chinese jujube (*Ziziphus jujuba* Mill.). Scientia Horticulturae **249**:263-270.

Wen Y, Liu H, Meng H, Qiao L, Zhang G, Cheng Z. 2022. *In vitro* induction and phenotypic variations of autotetraploid garlic (*Allium sativum* L.) with dwarfism. Frontiers in Plant Science **13**:917910.

Wendel JF. 2015. The wondrous cycles of polyploidy in plants. American Journal of Botany **102**(11):1753-1756.

WHO. 2023. Global Centre for Traditional Medicine. WHO, Switzerland. Available from https://www.who.int/initiatives/who-global-centre-for-traditional-medicine (accessed January 2023).

Wu Y, Li M. 2013. Induction of tetraploid plants of *Pogostemon cablin* (Blanco) and its quality evaluation. Pharmacognosy Journal **5**:281-285.

Xie Z, Huang H, Zhao Y, Shi H, Wang S, Wang TTY, Chen P, Yu LL. 2012. Chemical composition and anti-proliferative and anti-inflammatory effects of the leaf and whole-plant samples of diploid and tetraploid *Gynostemma pentaphyllum* (Thunb.) Makino. Food Chemistry **132**(1):125-133.

Xing SH, Guo XB, Wang Q, Pan QF, Tian YS, Liu P, Zhao JY, Wang GF, Sun XF, Tang KX. 2011. Induction and flow cytometry identification of tetraploids from seed-derived explants through colchicine treatments in *Catharanthus roseus* (L). G don. Journal of Biomedicine and Biotechnology **1**:10.

Xu L, Najeeb U, Naeem MS, Daud MK, Cao JS, Gong HJ, Shen WQ, Zhou WJ. 2010. Induction of tetraploidy in *Juncus effusus* by colchicine. Plant Biology **54**(4):659-663.

Xu Z, Chang L. 2017. Identification and control of common weeds. Springer, Singapore.

Yadav IS, Singh N, Wu S, Raupp J, Wilson DL, Rawat N, Gill BS, Poland J, Tiwari VK. 2022. Exploring genetic diversity of wild and related tetraploid wheat species *Triticum turgidum* and *Triticum timopheevii*. Journal of Advanced Research **48**:47-60.

Yan HJ, Xiong Y, Zhang HY, He ML. 2016. *In vitro* induction and morphological characteristics of octoploid plants in *Pogostemon cablin*. Breeding Science **66**:169-174.

Yan T, Chen M, Shen Q, Li L, Fu X, Pan Q, Tang Y, Shi P, Lv Z, Jiang W, Ma Y, Hao X, Sun X, Tang K. 2017. Homeodomain protein 1 is required for jasmonate-mediated glandular trichome initiation in *Artemisia annua*. New Phytologist **213**:1145-1155.

Yant L, Bomblies K. 2021. Genome management and mismanagement-cell-level opportunities and challenges of whole-genome duplication. Genes and Development **29**:2405-2419.

Yavari S, Omidbaigi R, Hassani ME. 2011. The effects of induction of autotetraploidy on the essential oil content and compositions of Dragonhead (*Dracocephalum moldavica* L.). Journal of Essential Oil Bearing Plants **14**(4):484-489.

Yunus A, Hikam MP, Widyastuti Y. 2018. Polyploid response of *Artemisia annua* L. to colchicine treatment. In IOP Conference Series: Earth and Environmental Science. **142**:(1)012020.

Zabka M, Pavela R, Slezakova L. 2009. Antifungal effect of *Pimenta dioica* essential oil against dangerous pathogenic and toxinogenic fungi. Industrial Crops and Products **30**(2):250-253.

Zahedi AA, Hosseini B, Fattahi M, Dehghan E, Parastar H, Madani H. 2014. Overproduction of valuable methoxylated flavones in induced tetraploid plants of *Dracocephalum kotschyi* Boiss. Botanical Studies **55**:22.

Zawiślak G, Nurzyńska-Wierdak R. 2017. Plant morphological parameters and yield of winter savory depending on the method of plantation establishment. Acta Scientiarum Polonorum Hortorum Cultus **16**(4):153-160.

Zelený V. 2012. Rostliny Středozemí. Second ed. Academia, Prague, Czech Republic.

Zeljkovic SĆ, Topčagić A, Požgan F, Štefane B, Tarkowski P, Maksimović M. 2015. Antioxidant activity of natural and modified phenolic extracts from *Satureja montana* L. Industrial Crops and Products **76**:1094-1099.

Zhang W, Hao H, Ma L, Zhao C, Yu X. 2010. Tetraploid muskmelon alters morphological characteristics and improves fruit quality. Scientia Horticulturae **125**(3):396-400.

Zhang H, An S, Hu J, Lin Z, Liu X, Bao H, Chen R. 2018. Induction, identification and characterization of polyploidy in *Stevia rebaudiana* Bertoni. Plant Biotechnology **35**(1):81-86.

Zhang K, Wang X, Cheng X. 2019. Plant Polyploidy: Origin, evolution, and its influence on crop domestication. Horticultural Plant Journal **6**:231-239.

Zhang CJ, Diao J, Wang Y, Zhang J, Yu J, Kim DS, Gao Y, Han M, Wu N, Zhang H, Liu R, Yan X. 2022. Hybridization rate and fitness of hybrids produced between the tetraploid *Camelina rumelica* and hexaploid *Camelina sativa*. Industrial Crops and Products **187**:115312.

Zheng K, Wang J, Zhang X, Hou Y, Dong W. 2022. Hazelnut germplasm innovation: induction of tetraploid hybrid hazelnut by colchicine. Plant Cell, Tissue and Organ Culture **152**(3)1-9.

Zu Y, Yu H, Liang L, Fu Y, Efferth T, Liu X, Wu N. 2010. Activities of ten essential oils towards *Propionibacterium* acnes and PC-3, A-549 and MCF-7 cancer cells. Molecules **15**(5):3200-3210.