CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Tropical AgriSciences

Oryzalin-mediated artificial polyploid induction in *Origanum vulgare* L.: a medicinal plant from the Lamiaceae family

MASTER'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled “Oryzalin-mediated artificial polyploid induction in *Origanum vulgare* L.: a medicinal plant from the Lamiaceae family” independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to the citation rules of the FTA.

In **Prague**, on **24. 4. 2024** .....

Mitali Paul

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Abstract

The current study marks a substantial breakthrough by reporting the first successful creation of autopolyploid *Origanum vulgare* L. (commonly known as oregano) varieties using oryzalin treatment under *in vitro* conditions. The main aims were to explore *O. vulgare*'s response to oryzalin treatment, with particular attention to polyploid formation, subsequent phenotypical and gene expression changes, as well as its value for agriculture and medicine. Six different treatments were applied to a total of 312 plants, resulting in only eleven autopolyploid variants. Among them, two autopolyploids (Poly C and Poly 12, based on their performance under *in vitro* conditions) were further chosen for subsequent analyses. The low success rate illustrated the challenges of inducing autopolyploidy with oryzalin in oregano. Survival rates reduced with increasing oryzalin concentration. The autopolyploid plants exhibited larger leaves and stomata, hypothetically indicating improved resource utilization and water conservation. Physiological parameters assessment also showed alterations in membrane integrity, photosynthetic efficiency, and nutrient uptake parameters in the polyploid genotypes. Additionally, gene expression analysis showed enhanced expression of *terpene synthase* and *cinnamate 4-hydroxylase* in polyploid plants, suggesting heightened production of essential oils and bioactive compounds. The practical implications of the current study include the potential for novel cultivars with improved traits for agriculture and higher medicinal value. However, it is also note-worthy that there are also some associated challenges such as low success rates and limited generalizability. Hence, there is a need for further optimization and validation. Based on these, the future research directions might involve combined transcriptomic-metabolomic studies to reveal the key genes and pathways associated with secondary metabolite biosynthesis. Additionally, we also propose considering the environmental effects, reference gene selection, and comparative efficacy studies, to augment the study's applicability and relevance.

Keywords: Flow cytometry; Gene expression; Induced polyploidy; *In vitro* propagation; Medicinal plants; Mint family.

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List of the abbreviations used in the thesis

cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
Fv	Variable fluorescence
Fm	Maximum fluorescence
gH+	Hydrogen ion conductance
MS medium	Murashige and Skoog medium
NPQt	Non-photochemical quenching of chlorophyll a fluorescence
Pi_Abs	Phosphate absorption capacity
LEF	Linear electron flow
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
S-phase	Synthesis phase
SPAD	Soil plant analysis development

1. Introduction and Literature Review

1.1 Introduction

Origanum vulgare L. (commonly known as oregano), is a woody perennial plant that is widely used in the culinary, medicinal, and cultural practices worldwide (Alekseeva et al. 2020). This herb belongs to the mint family Lamiaceae and contains small glands on its leaves and stem which produce volatile oil giving the sauce an aromatic flavor, giving it its unique fragrance. These oils contain important compounds such as menthol, thymol, and carvacrol, which are well-known for their therapeutic and scented qualities (Han et al. 2017).

In general, oregano plant contains small, dark green leaves (usually oval in shape and shadowy in colour). It produces clusters of pink or purple flowers in late summer. In most *Origanum* species, the basic chromosome number is 2n = 2x = 30. However, some populations might have either 28 or 32 chromosomes instead of 30 (Jedrzejczyk 2018; Bakha et al. 2017). The *Origanum* genus has an unclear taxonomy with a lot of genetic variation within the species. These variations might be due to the phenotypic characteristics and natural breeding between species which might/might not be closely related. With so many species, subspecies, varieties, and hybrids, it's hard to identify them correctly (Jedrzejczyk 2018). Oregano has numerous culinary uses and medical benefits. This species is primary in Mediterranean, Italian, and Mexican cuisines. Its leaves are widely used to enhance the flavor of pizzas, pasta sauces, salads, and marinades. Alongside this, dried oregano is also used as seasoning material and is used as a key ingredient in spice blends in many countries (Bower et al. 2019). In addition to its culinary uses, Oregano has numerous medical benefits. The essential oils are well-known for their antimicrobial, antiviral, and antifungal effects, besides having strong antioxidant, anti-inflammatory, antidiabetic, and cancer-suppressing properties (Leyva-López et al., 2017). The major breeding aims of this species includes:

1. Flavor enrichment via intensification of its aromatic compounds.
2. Increasing disease resistance via development of varieties that are resistant to common diseases and pests.
3. Breeding for elevated yields, profiting mainly the commercial growers.
4. Climate change resilient varieties.
5. Breeding to increase their harvesting efficiency.

While oregano is widely acknowledged for its miscellaneous useful properties (from antimicrobial to antioxidant effects), there is a significant research gap concerning its polyploidization. Despite its wide-ranging applications, till date, to the best of our knowledge, there have been no studies aiming on inducing polyploidization in *Origanum vulgare* (commonly known as oregano) via anti-mitotic agents like oryzalin. This gap in research highlights the potential for further exploration and the development of new polyploid oregano varieties, with superior traits. Hence, the current study can be considered a significant advancement by achieving the first successful creation of autopolyploid oregano varieties using oryzalin treatment under *in vitro* conditions. The results obtained from the study had successfully produced autopolyploid oregano genotypes using oryzalin treatment, demonstrating potential advantages such as larger leaves and increased gene expression for essential oils. Future studies with these varieties could open up new possibilities for improving oregano's agronomic characteristics and bioactive compound profiles.

1.2. Taxonomical description

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Asteridae

Order: Lamiales

Family: Lamiaceae

Genus: *Origanum*

Species: *O. vulgare*

Binomial name: *Origanum vulgare* Linnaeus

Figure 1: *Origanum vulgare* L.

Source: https://www.needpix.com/photo/1087352/oregano-spice-aromatic-herbs-mediterranean-culinary-herbs-edible

Origanum vulgare is a multi-purpose herb in the mint family Lamiaceae, mainly known for its flavorful leaves and culinary uses (Figure 1). The Lamiaceae family consists of a wide-range of plants (with over 7,000 species) and is one of the most important families of flowering plants. Some key characteristics associated with the Lamiaceae family include: (a) most of the members of this family contain oil-producing glands (volatile in nature) on their leaves and stems; (b) this family have typical square stems ("quadrate" stems); (c) the leaves are arranged in pairs opposite each other along the stem, and (d) the flowers have bilateral symmetry, and are arranged in spikes, racemes, or panicles (Raja 2012).

1.3 Botanical and morphological description

Oregano is a perennial herbaceous plant (lives for more than two years) with small purple flowers that typically grows to about 1 to 2 feet tall (Figure 2). Despite being a native to the temperate areas (western and southwestern Eurasia and the Mediterranean region), this plant can also be grown in colder climates. However, they habitually does not survive the winter (Alekseeva et al. 2020). The leaves are harvested prior to the flower blooming. In general, Oregano plants have small, oval-shaped leaves that usually grows 1 to 4 cm in length (Figure 2). The leaf colour usually varies between the upper and lower surfaces. The upper surface is typically dark green in color whereas the underside is comparatively lighter green in colour. Oregano leaves are covered with tiny,

glandular hair-like structures called trichomes and emit a strong aroma when crushed. The stems of oregano are woody at the base and herbaceous towards the top. Like the leaves, the stems of oregano can also consist of trichomes (Shafiee-Hajiabad et al. 2014). However, the stem trichomes are typically less dense than those on the leaves. Moreover, the oregano stems have a typical square-shape, a general characteristic feature of the mint family. Oregano has a fibrous root system that helps it spread and anchor itself in the soil. The roots can reach a maximum of 45 cm deep. Like most of the dicotyledonous plants, the roots of oregano start out with a taproot as the primary root, then lateral secondary roots grows out of it, hence it appear to be fibrous (Węglarz et al. 2020).

Figure 2: Pictures of leaves, flowers, and roots of *Origanum vulgare* L.
Source: https://materiamedicaresource.wordpress.com/2018/08/13/origano/

1.4 Polyploidization in crop improvement and breeding

1.4.1 Introduction to polyploidization

Polyploidization (having more than two full sets of chromosomes in a cell) is an important phenomenon in the field of crop improvement and breeding. It is known to play vital roles in increasing genetic diversity and introducing novel traits. This increased diversity allows the polyploid plants to adapt easily to various challenges, including diseases, pests, extreme temperatures, drought, and salinity. In general, polyploids can be divided into four main types: autopolyploids, allopolyploids, autoallopolyploids, and segmental allopolyploids. The autopolyploids contain multiple sets of chromosomes, but from a single species. Hence, they might produce cells with the improper number of chromosomes. As a result, the progenies are often sterile and have lower fitness (Trojak-Goluch et al. 2021). In contrast, the allopolyploids are hybrids of closely or distantly related species and both the parent species contribute to the extra chromosome sets (Trojak-Goluch et al. 2021). Some important allopolyploids used in agriculture and industry include *Gossypium hirsutum* L., *Nicotiana tabacum* L. (Berbeć & Doroszewska 2020) and *Saccharum officinarum* L. (Trojak-Goluch et al. 2021). In the case of autoallopolyploids the allopolyploids undergo genome doubling. Hence, they can be considered as hybrids with genome doubled after formed from the combination of two different species. Another category of polyploids is "segmental allopolyploids". These polyploids might form as a result of either disomic inheritance or tetrasomic inheritance. In both the cases, the recombination occurs within the subgenomes. Hence, the segmental allopolyploids can be considered as intermediate between allopolyploids and autopolyploids (Mason & Wendel 2020).

1.4.2 Effects on the plant morphological and physiological changes

Increased ploidy levels might affect various important parameters within the plants cells, thus resulting in shifts in their anatomical structures. One such substantial change can be the enlargement of cell size and subsequent amendments in the surface area to volume ratio (Doyle & Coate 2019). Besides the increase of cell size, polyploidization can induce several other changes such as increase in the size of the cell nucleus, altered cell cycle lengths, leaf shape, flower size, root structure, altered flowering time, fertility, and seed production capacity. For example, in a study conducted with *Mentha spicata* L., Bharati et al (2023a) discovered significant morphological differences between the control and polyploid plants. They found that some of the polyploids had more shoots, doubled leaf area and thicker leaves and stems, compared to the control (Bharati et al. 2023a). In another study conducted on the effects of polyploid induction in *Salvia*

officinalis L., the authors found that the tetraploid plants had increased leaf length, leaf width, plant height, number of leaves, number of nodes, internodes length and stomata size (along with increase in other parameters) and decreased stomata count as compared with diploid ones (Hassanzadeh et al. 2020). In another more recent interesting study, the authors aimed to explore the growth, physiology, and stomatal parameters of polyploids cultivated under ice age, present-day, and future CO_2 concentrations (200, 400, and 800 ppm, respectively). Interestingly, they found that polyploids grew comparatively better than diploids even at low, glacial CO_2 concentrations. However, there were several associated fitness costs such as decreased water-use efficiency and a reduced growth benefit (Šmarda et al. 2023). Inducing tetraploidy in *Erysimum cheiri* (L.) Crantz had also resulted in increased flower count, longer longevity, larger size, and extended flowering duration (Fakhrzad et al. 2023).

1.4.3 Effects on the amount and composition of plant secondary metabolites

Increased ploidy levels generally lead to an increase in the copy number of the individual genes, which eventually affects the enzymes and metabolic pathways in plants. For example, in a recent study with *Lonicera caerulea* L., the authors showed a positive relationship between the ploidy levels and substances like quinic acid and sorbitol (Li & Hoshino 2024). Similarly, in another study with *Thymus vulgaris* L., the authors found that the induced tetraploid plants produced 64.7% more essential oil compared to diploid plants, with increases of 40.9% in thymol and 18.6% in carvacrol content (Mohammadi et al. 2023). In another two independent studies with *M. spicata* and *Melissa officinalis* L., similar results were obtained. The authors found that the induced polyploid genotypes of these plants produced higher levels of essential oils compared to their control counterparts (Bharati et al. 2023a,b).

1.4.4 Effects on the plant stress resistance

In addition to the plant morphological, physiological, and secondary metabolites changes, polyploid plants are also known to have enhanced ability to tolerate various stresses, such as drought, salinity, and extreme temperatures. This increased stress resistance might be attributed to several polyploidy-associated factors such as genetic redundancy (act as a buffer against mutations), improved water-use efficiency or altered hormone signaling or metabolic adaptations.

Additionally, the polyploid genotypes may also have variations in the gene expression patterns or regulatory networks leading to the enhancement of the stress response pathways (Li et al. 2024). For example, tetraploid *E. cheiri* plants demonstrated superiority (compared to diploid counterparts) under water stress conditions owing to their enhanced osmotic adjustment, stronger antioxidant enzyme activity, and improved non-enzymatic defense systems. Moreover, changes in stress hormone levels and the presence of specific phenolic compounds were also identified. The authors hypothesized that these factors had likely contributed to their superior stress resistance (Fakhrzad et al. 2023). In another study, the authors found that a 12X *Dianthus broteri* Boiss. & Reut. cytotype displayed superior stress tolerance, maintaining gas exchange and water status under extreme temperatures by adjusting the gene expression and photochemical integrity (López-Jurado et al. 2024).

1.4.5 Antimitotic agents as polyploidization induction agents

Anti-mitotic agents are known for their vital roles in interrupting mitosis and leading to abnormal chromosome segregation. In general terms, anti-mitotic agents induce endoreduplication. Endoreduplication happens when a cell undergoes additional rounds of DNA synthesis during the S-phase. In such cases, the cell does not proceed to mitosis or cytokinesis. This results in multiple chromatids for each chromosome and formation of polyploid cells. Some commonly use anti-mitotic agents include:

- **Colchicine**: It is derived from the plant *Colchicum autumnale* L. this anti-mitotic agent inhibits microtubule polymerization. As a result, the disruption of spindle formation occurs during cell division. Colchicine is the most commonly used mitotic inhibitor in the *in vitro* studies (Leung et al. 2015). Colchicine also has several advantages such as excellent water-solubility, good heat-stability and, can be autoclaved. Moreover, colchicine is easily applied to plant tissues. However, it has several disadvantages too. Colchicine is potentially toxic to humans and its binding affinity to plant microtubules is also low, i.e. higher concentrations are needed to work effectively (Touchell et al. 2020).

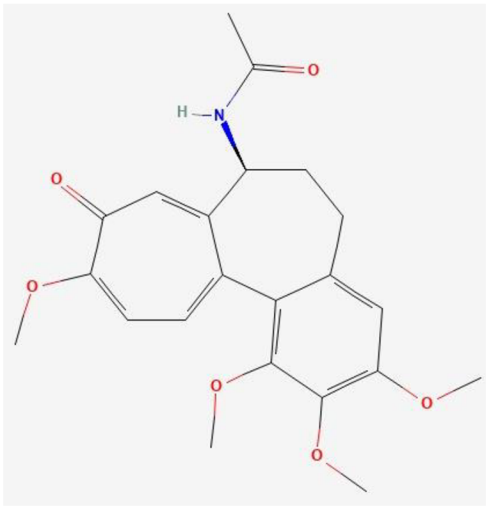

Figure 3: 2D Structure of colchicine (PubChem CID: 6167). Source: https://pubchem.ncbi.nlm.nih.gov/

- **Oryzalin**: Oryzalin is a synthetic anti-mitotic agent. This chemical is known to induce polyploidy by disrupting microtubule assembly. It inhibits tubulin polymerization, disrupts the mitotic spindle, and subsequently prevents the cell division. This compound is soluble in organic solvents and can be considered a relatively safer than colchicine. However, it has a few drawbacks including lower binding affinity to plant microtubules and needs higher concentrations for effectiveness. It can be used similar to colchicine to treat plant tissues (Touchell et al. 2020).

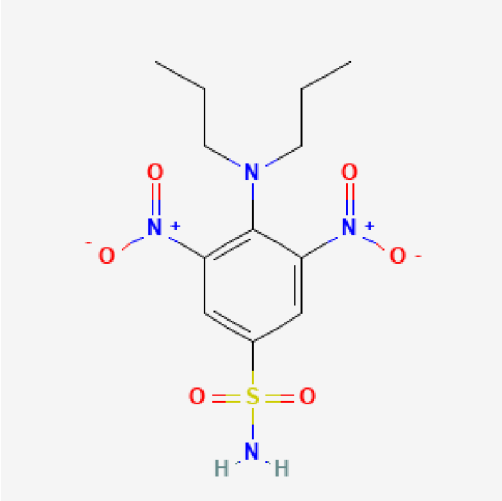

Figure 4: 2D Structure of oryzalin (PubChem CID: 29393). Source: https://pubchem.ncbi.nlm.nih.gov/

Apart from colchicine and oryzalin, there are other methods of inducing polyploidization. These includes nitric oxide treatment, dimethyl sulfoxide (DMSO) treatment, high temperature treatment, electrical stimulation, and gamma irradiation. Nitrous oxide is usually used in gas form, and it interferes with chromosome separation (interacts with α-tubulin) during cell division (Kitamura et al. 2009). On the other hand, DMSO disrupts cell membranes and affects the cell division. However, DMSO is often used in combination with other agents to boost their effectiveness (Dhooghe et al. 2011). Electric pulses can disrupt cell membranes and promote polyploidy (Rauf et al. 2021).

1.5 Importance of gene expression studies in *in vitro* polyploidization experiments

Gene expression which results in the subsequent production of proteins is the underlying link connecting the genotype to the phenotype (Romero et al. 2012). Hence, understanding the changes in gene expression resulting from polyploidization can be crucial for understanding the exact mechanisms underlying polyploid formation and its effects on the development, adaptation, and evolution of the organism. Gene expression studies can be essential while understanding the complex relationship between the genome duplication and gene regulation, improving breeding programs, understanding genome evolution and adaptation, alongwith discovering the possible consequences of polyploidy on human health and disease (Varshney et al. 2005). Polyploidization events might lead to significant changes in gene expression patterns due to alterations in gene dosage, epigenetic modifications, and genome restructuring. Gene expression studies allow researchers to study these associated changes. By comparing gene expression profiles between the polyploid and their diploid counterparts, researchers can pin down the specific genes and pathways that might be affected by polyploidization (Osborn et al. 2003; Chen 2007). Understanding these molecular mechanisms can be extremely essential while studying how polyploidy might influence traits such as growth rate, stress tolerance, and reproductive success. Moreover, it will also provide novel perceptions into the evolutionary forces that shape the polyploid genomes and force speciation.

Additionally, the gene expression studies can be used to improve the existing polyploid breeding programs (Bourke et al. 2018). In most cases, polyploid plants have shown advantages over their diploid counterparts, featuring a wide-range of desirable traits like boosted vigor, larger

fruits, and improved disease resistance (Ruiz et al. 2020). The gene expression studies can help the plant breeders to identify the candidate genes responsible for these traits and develop approaches to incorporate these beneficial traits into top-performing varieties. Correlating the gene expression patterns with the observable phenotypic traits might help the researchers to discover novel molecular markers associated with desirable traits (Vuylsteke & Van Eeuwijk 2008). Moreover, these studies might also be beneficial while improvising the existing markers and then these markers can be used for marker-assisted selection to improve the breeding process. Likewise, comprehending the genetic basis of polyploid-specific traits can help breeders develop new cultivars with improved agronomic performance and quality.

2. Aims of the Thesis

The general goal of this thesis was to assess the morphological and molecular differences between diploid (2n = 2x = 30) and autotetraploid (2n = 4x = 60) *Origanum vulgare* plants. The autotetraploid plants were produced through *in vitro* induced mitotic polyploidy using oryzalin as an antimitotic agent.

The specific aims of this thesis are:

- The primary aim is to develop autopolyploid *Origanum vulgare* L. using anti-mitotic agent.
- To explore the impact of induced polyploidization on oregano.
- Investigate the gene expression patterns of the two key genes involved in secondary metabolite biosynthesis, influenced by induced polyploidization.
- Offer practical implications for the agricultural and medicinal applications of oregano based on our study's findings.

The hypotheses formulated based on the aims are as follow:

- **H1:** Oryzalin treatment can successfully create polyploid oregano plants.
- **H2:** The induced polyploidization have effects on the physical characteristics of the *Origanum vulgare* L., leading to changes in plant morphology compared to their control counterpart.
- **H3:** Induced polyploidization influences the gene expression profiles of secondary metabolite biosynthesis.

3. Methods

The below-described methods was pre-optimized in the Laboratory of Plant Tissue Cultures at the Faculty of Tropical Agrisciences, Czech University of Life Sciences Prague, Czech Republic and already used previously for the propagation of *M. spicata* and *M. officinalis* (Bharati et al. 2023a,b).

3.1 Plant material collection and *in vitro* multiplication

Explants of *Origanum vulgare* (2n = 2x = 30) were obtained from the medicinal plant collection at the botanical garden of the Faculty of Tropical Agrisciences, Czech University of Life Sciences Prague, Czech Republic. These samples were grown under greenhouse conditions. The average temperature was maintained at 23°C and relative humidity ranging from 70% to 80%. Stems with a minimum of 5 nodes were selected for the experiments. First the stems were washed under running tap water for 10 mins. Thereafter, the nodal segments were soaked in 70% ethanol (2 minutes), and then they were treated with 1% sodium hypochlorite solution (commercial bleach-SAVO) containing tween 20 (a few drops). Briefly, the samples were dipped and continuously stirred in this solution for at least 10 minutes. Subsequently, they were washed three times with sterile distilled water and relocated to the laminar airflow hood for inoculation into a suitable growing media. Initially the media was prepared in 250 mL Erlenmeyer flask with approximately 50 mL of MS basal medium containing 3% sucrose, 0.8% agar, and the pH was adjusted to 5.7 ± 0.1. The cultures were kept in a growth room at 24/20± 1 °C (day/night) with a relative humidity of 65% to 70%. The photoperiod was set at 16/8 h (light/dark) under white, fluorescent lamps providing 3800 lux (51.3 µmol m^{-2} s^{-1}). Plants that grew were propagated by transferring nodal segments every 14 days until enough plants were available for polyploid induction.

3.2 Polyploidy induction

The 10-15 mm long nodal segments from the *in vitro* plants were re-cultured on MS medium for 48 hours before treatment. Thereafter, oryzalin solutions at three different concentrations (20, 40, and 60 µM) were prepared in 1% DMSO and applied to the segments in a sterile laminar flow box. Six treatments (T1-T6) with different oryzalin concentrations and exposure times were tested: T1 (20 µM for 24 h), T2 (20 µM for 48 h), T3 (40 µM for 24 h), T4 (40 µM for 48 h), T5 (60 µM for

24 h), and T6 (60 µM for 48 h), using at least 52 segments per treatment. After the treatment, the nodal segments were washed thrice with sterile water, then cultured on MS medium for six months with monthly subculturing. Ploidy levels were checked every three months using a flow cytometer (Partec GmbH, Münster, DE).

3.3 *Ex vitro* transferring of the diploid control and the polyploid plants

After maintaining for at least six months under *in vitro* conditions, the treated plants were moved to the greenhouse with temperatures averaging 24°C and relative humidity ranging from 60% to 70%. They were planted in small pots (5 × 5 cm^2) filled with a 3:1 mix of soil and perlite. The plants were covered with transparent polythene for seven days for primary hardening. After this, they were repotted into larger pots and kept in the climate chamber (Snijders Produktie B.V., Tilburg, Netherlands) containing a similar average temperature and relative humidity to that of the greenhouse. The polythene covers were removed. The plants were occasionally watered and supervised. Two polyploids (based on their performance under *in vitro* conditions) were further chosen for subsequent analyses.

3.4 Flow-cytometry analysis

Flow cytometry was conducted following the method outlined by Bharati et al. 2023b. Briefly, a leaf section of approximately 1cm^2 was minced with a razor blade with 1 mL of Otto I buffer (0.1M $C_6H_8O_7$, 0.5% Tween20) in a petri dish. The crude suspension was subsequently filtered through a 50 µm nylon mesh. To this filtrate, 1 mL of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12H_2O$) containing 2 µg/mL of DAPI was added. Samples were then analyzed using a Partec PAS flow cytometer (Partec GmbH, Münster, DE). DNA content was recorded as a histogram using Flomax software (Version 2.3).

3.5 Comparative trait analysis of diploid and polyploid plants

The morphological characteristics of both diploid control (2n = 2x = 30) and autopolyploid (2n = 4x = 60) genotypes were studied to evaluate the impact of polyploidization. Parameters assessed included the number of shoots, shoot length, number of nodes per shoot, internodal distance, number of leaves per shoot, leaf area, leaf thickness, and stem thickness. Additionally, the

photosynthetic parameters were also assessed. The photosynthetic parameters include membrane permeability (gH+) assessment, linear Electron Flow (LEF) estimation, soil plant analysis development (SPAD) analysis, photoprotection (NPQt) estimation, photosynthetic efficiency (Fv/Fm) evaluation and phosphate absorption capacity (Pi_Abs) assessment. To evaluate the photosynthetic performance of the diploid and tetraploid genotypes were evaluated using a portable FluorPen FP110 (PSI, Czech Republic) and a MultispeQ V 2.0 device linked to the PhotosynQ platform (www.photosynq.org), both set to their default configurations. Additionally, the length, width, and density of stomata, were evaluated using the nail polish impression technique (Bharati et al. 2023b). The underside of the leaves was coated with clear nail polish and left to dry. Imprints were then taken using transparent tape and placed on a glass slide for measurement of stomatal dimensions and density under a light microscope at 60× magnification.

3.6 Gene expression analyses

Total RNA was isolated from plant tissues (±100 mg) using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The quality and integrity of the RNA were evaluated with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and 1.2% agarose gel electrophoresis. TURBO DNA-free™ Kit (Invitrogen, Waltham, MA, USA) was used for cDNA synthesis, following the manufacturer's protocol. cDNA synthesis was done using 1µg (10µL was used 100ng/µL) of high-quality, gDNA-free RNA as the template. The details of the primers used in this study can be found in table 1. The primers were designed using Primer3 software [version 4.1.0)]. All the designed primers were initially tested using a standard PCR (C1000 thermocycler, Bio-Rad, Hercules, CA, USA) and were confirmed by 1.8% agarose gel electrophoresis. The CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) was used for the qRT-PCR analysis. The reaction mixture was designed according to Bharati et al. 2023c with approximately 10 ng of cDNA. The thermocycler was set with an initial denaturation at 95 °C for 5 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Melting curves were obtained through a stepwise heating from 60 to 95 °C. *Elongation factor 1-α* was chosen as the reference gene/s (Crocoll et al. 2010), and the expression levels of the *terpene synthase* and *cinnamate 4-hydroxylase* genes were calculated using the $2^{-\Delta\Delta Ct}$ method. The experiment was done with five biological replicates.

Table 1: List of primers used in this study.

Gene name	Primer sequence (5' - 3')		Annealing temperature
Terpene synthase	Forward primer	TGATTGGGTTGTGAGCGAAC	60 °C
	Reverse primer	TTGCGGAGTTCTTGAAAGGC	
Cinnamate 4-hydroxylase	Forward primer	GTGGAGGACGTGAAGAGGAA	
	Reverse primer	CGACTCCTCTCCCCATTCAG	
Elongation factor 1-α	Forward primer	CTCCAGTTCTTGATTGCCACAC	
	Reverse primer	GCTCCTTTCCAGACCTCCTATC	

3.7 Statistical analysis

The obtained results were compared between the control and the induced autopolyploids using Student's t test at $p < 0.05$ in Microsoft Excel for Office365 software package. The graphs were also designed using Microsoft Excel for Office365.

4. Results

4.1 Survival and polyploid induction rate of *Origanum vulgare* following oryzalin treatment

The effects of different concentrations of oryzalin on the survival rate and autopolyploid induction rate in *O. vulgare* is shown in table 2. Fifty-two plants were used for each concentration. In the control plants (diploid), the survival rate is 100% with no variability and the autopolyploid induction rate is not applicable (0%). This shows that the control group (diploid) can serve as the perfect standard against which the other treatments can be compared. The high survival rate indicates that there are no influence of any external factors on the control group. In the current experiment, we have used three different oryzalin concentrations: 20 µM, 40 µM and 60 µM. Each concentration was tested for two different time durations: 24 hours and 48 hours. Reduced survival rate compared to the control group was observed consistently with all the oryzalin treatments. However, the survival rate varied from a low of 5.77% (60 µM, 48 hours) to a high of 92.31% (T2, 20 µM, 48 hours).Overall, the survival rates reduced with higher concentrations of oryzalin and longer treatment times. Only treatment T2 (20 µM, 48 hours) showed some level of autopolyploid induction (11 autopolyploids were obtained out of 52 tested plants). The other treatments did not induce polyploidy based on our experiment. This indicates that the success rate of autopolyploid induction in this species was generally low (only 21.15% in T2) across the various treatments.

Table 2: Polyploidization and the viability of *Origanum vulgare* nodal segments exposed to oryzalin.

Treatment	Oryzalin concentration (μM)	Treatment time (hours)	Survival rate (%)	Polyploid induction rate (%)
Control	0	not applicable	100 ± 0.0	not applicable
T1	20	24	51.92 ± 0.50*	0 ± 0.0
T2	20	48	92.31 ± 0.26*	21.15 ± 0.4*
T3	40	24	36.54 ± 0.49*	0 ± 0.0
T4	40	48	34.62 ± 0.48*	0 ± 0.0
T5	60	24	40.38 ± 0.50*	0 ± 0.0
T6	60	48	5.77 ± 0.24*	0 ± 0.0

The "*" indicates significant at 5% significance level (compared to the control). The sample size (n) for each treatment is 52.

4.2 Control vs Polyploids: phenotypic and flow-cytometry analysis

The flow cytometry results have revealed the presence of polyploidy in our samples (Figure 5). In the control group, the flow cytometry results showed a single peak at 100, which is consistent with our expectations. This doubling of the DNA content in the autopolyploid samples indicates a significant change in the genetic material compared to the control. Together with this finding, we have also observed phenotypic differences among the individuals (Figure 6).

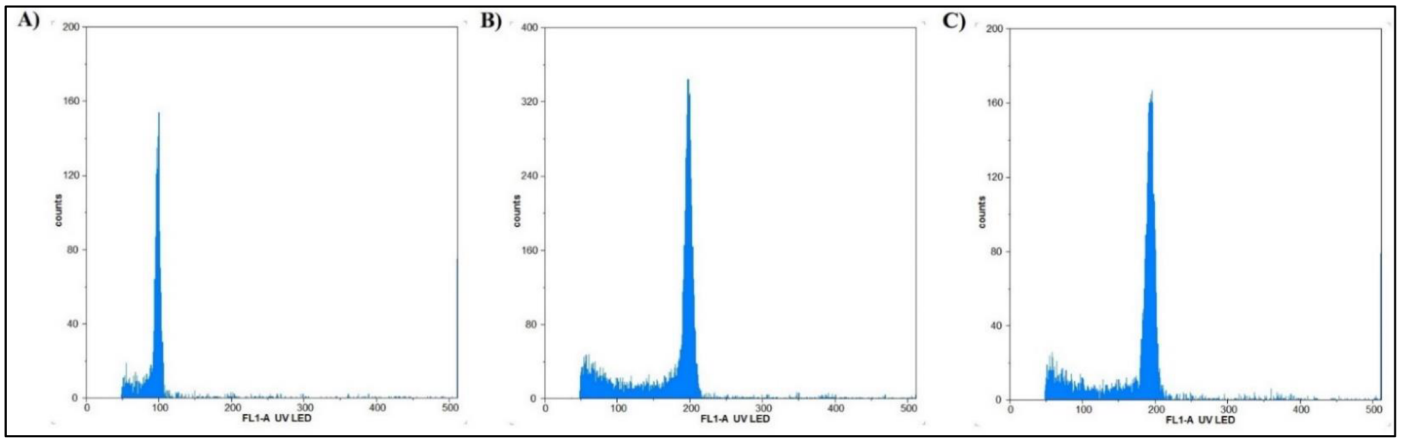

Figure 5: Flowcytometry analysis results. (A) Control plants (2n = 2x = 30), (B) Autopolyploid plant (Poly C; 2n = 4x = 60), (C) Autopolyploid plant (Poly 12; 2n = 4x = 60).

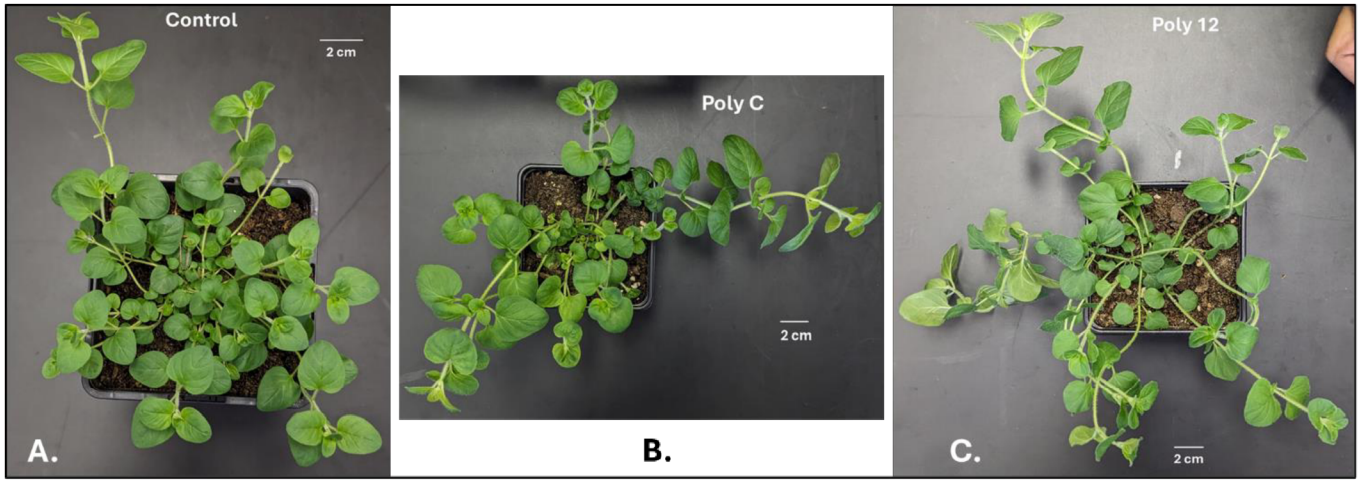

Figure 6: Phenotype results. (A) Control plants (2n = 2x = 30), (B) Autopolyploid plant (Poly C; 2n = 2x = 30), (C) Autopolyploid plant (Poly 12; 2n = 2x = 30).

4.3 Control vs polyploid plants: growth characteristics

4.3.1 Variations in the leaf size, width, and length

Figure 7 shows the leaf size variation results between the control and the autopolyploid plants (Poly C & Poly 12). In general, the autopolyploid plants exhibited larger leaf sizes compared to the control, with Poly C showing the most significant difference. Five representatives (n = 5) were considered and compared for these analyses. In the control plants, the leaf length varied from 9.18 cm to 5.98 cm and the leaf width varied from 10.96 cm to 7.41 cm. The control plants have an average leaf width of ~ 9.4 cm (± 1.3) and an average leaf length of ~7.5 cm (± 1.3). The leaf width in the Poly C plants ranged from approximately 11.20 cm to 12.61 cm while the leaf length varied from 10.64 cm to 13.71 cm. in case of the Poly C plants, the leaf width varied from 9.38 cm to 12.07 cm, and the leaf length varied from 8.66 cm to 12.41 cm. The results for the variations in the leaf width and length variation can be found in the Figure 8.

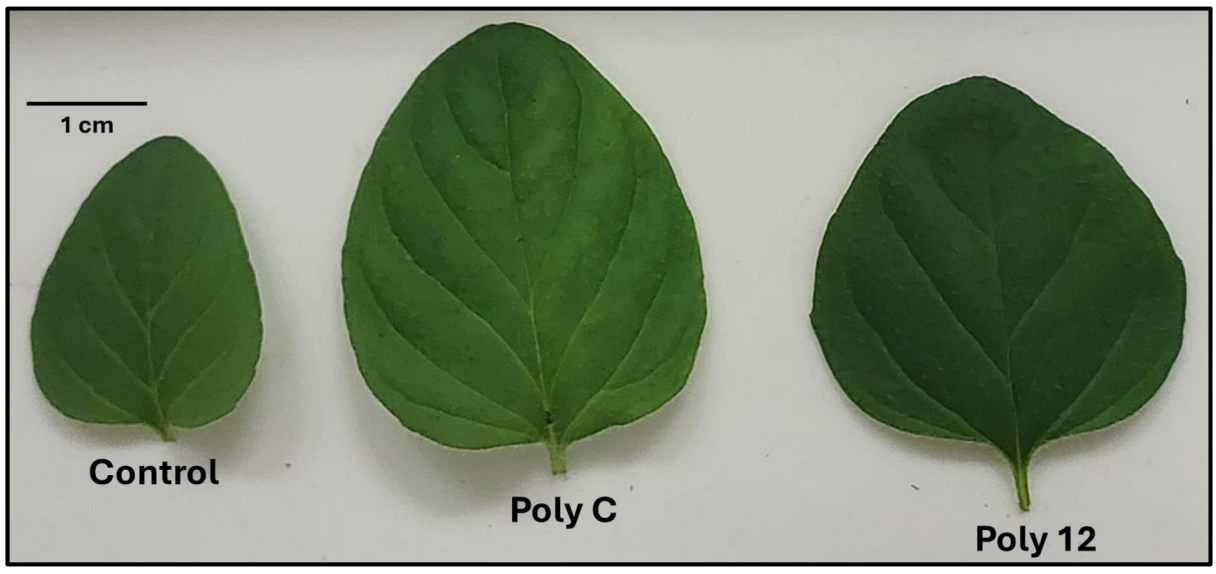

Figure 7: Leaf size variation results between the control (2n = 2x = 30) and the autopolyploid plants (Poly C & Poly 12; both have 2n = 4x = 60).

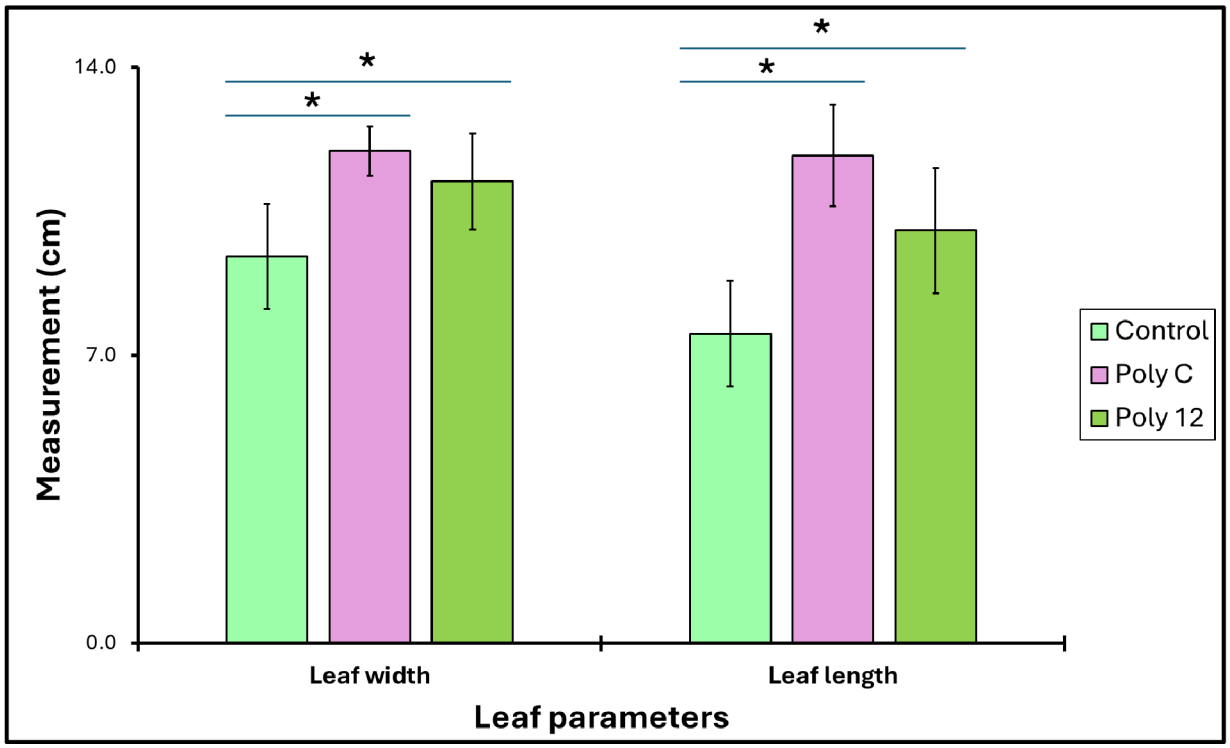

Figure 8: Leaf width and length variation results between the control and the autopolyploid plants (Poly C & Poly 12). The "*" indicates significant at 5% significance level (compared to the control). The sample size (n) is five.

4.3.2 Variations in the number of shoots and the shoot length

Figure 9 shows the number of shoots and the shoot length variation results between the control and the autopolyploid plants (Poly C & Poly 12). Even though the Poly C plants have a higher shoot number compared to the control, it's not statistically significant ($p=0.19$). On the other hand, the Poly 12 plants have the same shoot number as the control. While the shoot number between the control and the polyploid plants doesn't differ significantly, the shoot length does. Specifically, the autopolyploid plants have longer shoots than the control plants (statistically significant), with Poly 12 having the longest shoots. The average shoot length for the control plants is 15.18 cm (± 1.91). The average shoot length for Poly C increased by ~1.3X (compared to the control) to 19.13 cm (± 3.59). On the other hand, the average shoot length for Poly 12 is 21.28 cm (± 1.11), which is also higher than the control group.

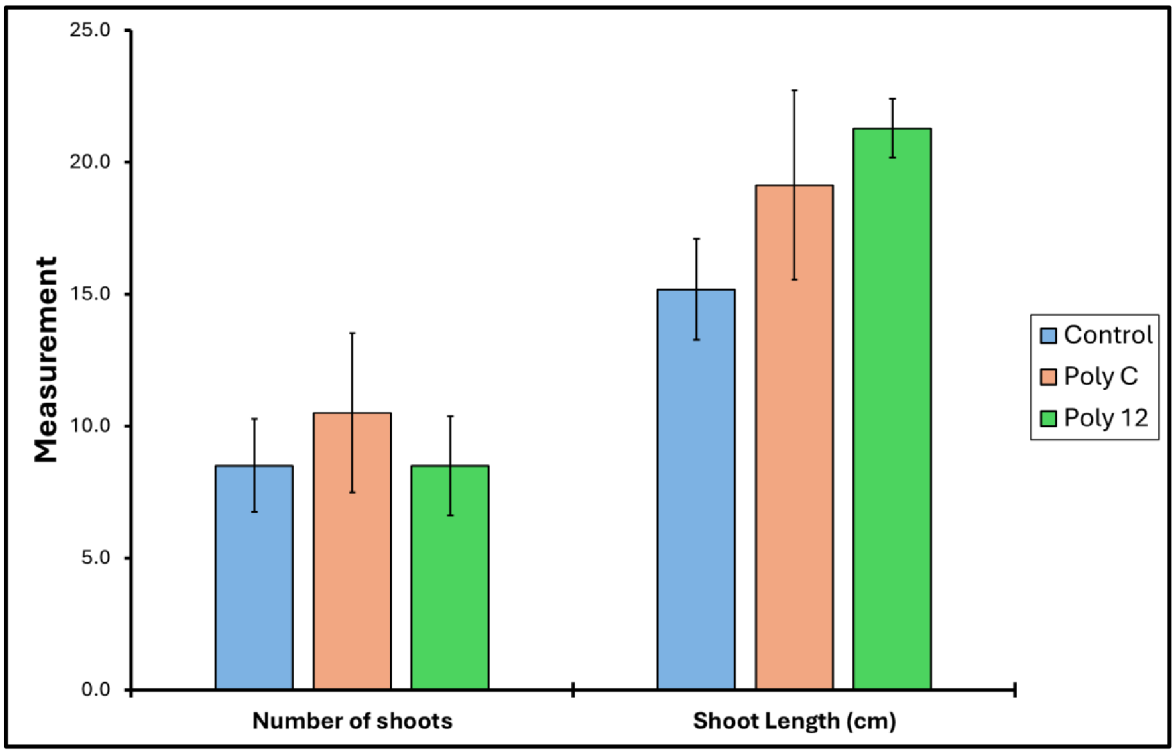

Figure 9: Variations in the number of shoots and the shoot length between the control and the autopolyploid plants (Poly C & Poly 12). The "*" indicates significant at 5% significance level (compared to the control). The sample size (n) is 6.

4.3.3 Variations in the leaf and stem thickness

Figure 10 shows the variations in the leaf and stem thickness between the control and the autopolyploid plants (Poly C & Poly 12). The leaf thickness showed significant differences between the control and the autopolyploid plants. The average leaf thickness of the control plants was 0.37 mm (± 0.05), whereas the average leaf thickness of the Poly C and the Poly 12 plants were 0.43 mm (± 0.08) and 0.43 mm (± 0.06), respectively. The stem thickness was not significantly different between the Control and Poly C plants but showed a highly significant difference between the Control and Poly 12 plants. The average stem thickness of the control plants was 1.49 mm (± 0.07), whereas the average stem thickness of the Poly C and the Poly 12 plants were 1.44 mm (± 0.34) and 1.78 mm (± 0.12), respectively.

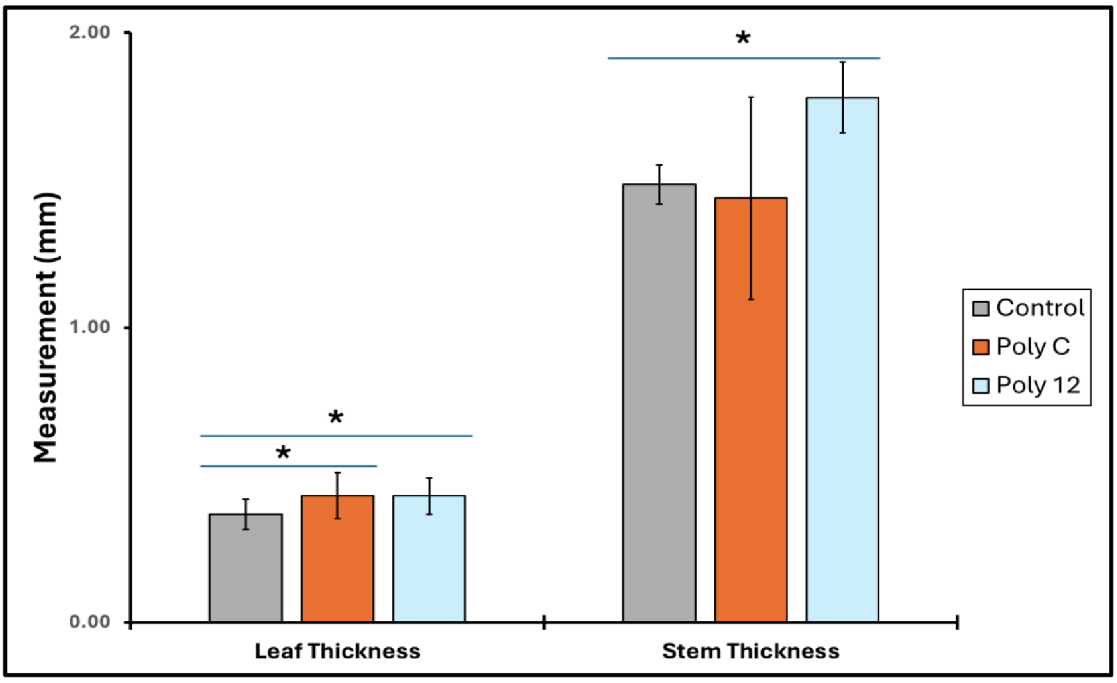

Figure 10: Variations in the leaf and stem thickness between the control and the autopolyploid plants (Poly C & Poly 12). The "*" indicates significant at 5% significance level (compared to the control). The sample size (n) is 10.

4.3.4 Stomatal size and frequency: control vs polyploid

Both the autopolyploid genotypes (Poly C and Poly 12) showed a significant reduction in the average number of stomata per magnification field compared to the control, with Poly 12 having the most significant decrease (more than 2X). Poly 12 genotype has a significantly longer stomatal length compared to the control, while Poly C shows a slight increase but not significant. In the case of stomata width, both the autopolyploid genotypes have wider stomata compared to the control. Poly C had a slightly wider width compared to Poly 12. Hence, we can say that polyploidization might have influenced the stomatal characteristics in *O. vulgare*, leading to changes in stomatal number, length, and width. The details of the stomatal parameters can be found in figure 11 and table 3.

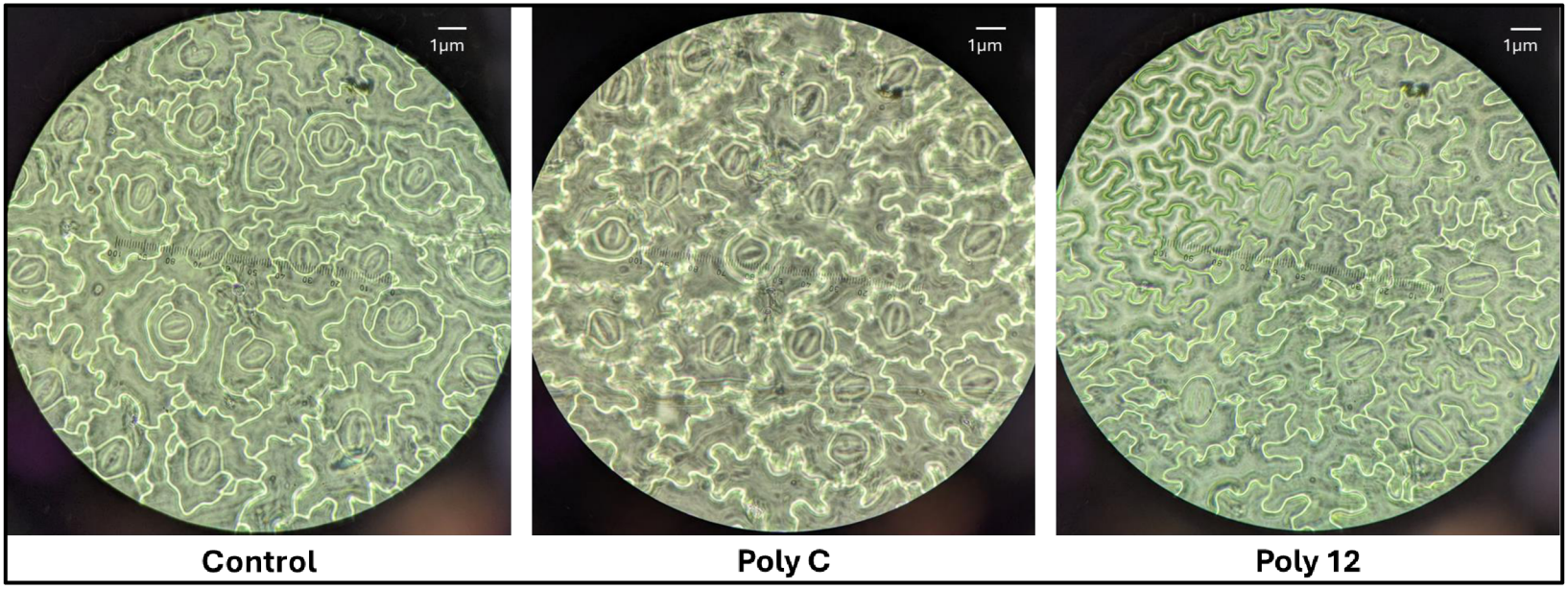

Figure 11: Stomatal size and frequency comparison between the control and the autopolyploid plants (Poly C & Poly 12).

Table 3: Stomatal descriptions between the control and autopolyploid genotypes of *Origanum vulgare*.

Variant	Average number of stomata per magnification field (60×)	Stomata length (cm)	Stomata width (cm)
Control	20.63 (± 0.74)	4.24 (± 0.80)	3.72 (± 0.49)
Poly C	19.88 (± 0.35)	4.63 (± 0.63)	4.21 (± 0.54)*
Poly 12	9.75 (± 0.71)	5.35 (± 0.81)*	4.26 (± 0.41)*

The "*" indicates significant at 5% significance level (compared to the control).

4.3.5 Chlorophyll fluorescence kinetics: control vs polyploid

The gH+ is a measure of hydrogen ion conductance or proton flux across the membrane. Higher values indicate increased membrane permeability. Based on our analyses, we observed that the Poly C plants have the highest membrane permeability value, compared to the control and Poly 12 (Table 4). The parameter LEF stands for Linear Electron Flow. It is a measure of energy flow in a photosynthetic system. A higher value indicates better photosynthesis. Poly 12 exhibited a slightly higher LEF value than the control and Poly C. However, no significant differences were detected in any of the autopolyploids (Table 4). The control plants have the highest SPAD value, followed by Poly 12 and then Poly C. Higher SPAD value is an indication of increased chlorophyll content, which is crucial for photosynthesis (Figure 12). Poly C exhibited the highest NPQt value. This suggests that Poly C plants can better dissipate excess light energy as heat (compared to the control plants), a protective response against photodamage (Table 4). No significant differences in the Fv/Fm were obtained. All groups showed similar Fv/Fm values. This suggests a comparable photosynthetic efficiency across the variants and the polyploidization have no detrimental effects on the maximum quantum yield of Photosystem II (Table 4). Poly 12 had the highest phosphate absorption capacity value, suggesting a potentially better ability to absorb and utilize phosphate compared to the control and Poly C. This could imply that Poly 12-treated plants might have an advantage in nutrient uptake and utilization (Table 4).

Table 4: Chlorophyll fluorescence metrics analysis between the control and autopolyploid genotypes of *Origanum vulgare*.

Variant	gH+	LEF	NPQt	Fv/Fm	Pi_Abs
Control	127.02 (± 82.43)	26.01 (± 4.91)	0.72 (± 0.18)	0.84 (± 0.01)	5.42 (± 2.13)
Poly C	157.91(± 81.46)*	25.33 (± 2.30)	0.78 (± 0.20)*	0.84 (± 0.01)	5.19 (± 1.55)
Poly 12	125.67(± 38.94)	27.37 (± 1.75)	0.72 (± 0.06)	0.84 (± 0.00)	5.64 (± 0.95)

The "*" indicates significant at 5% significance level (compared to the control). The sample size (n) is 10.

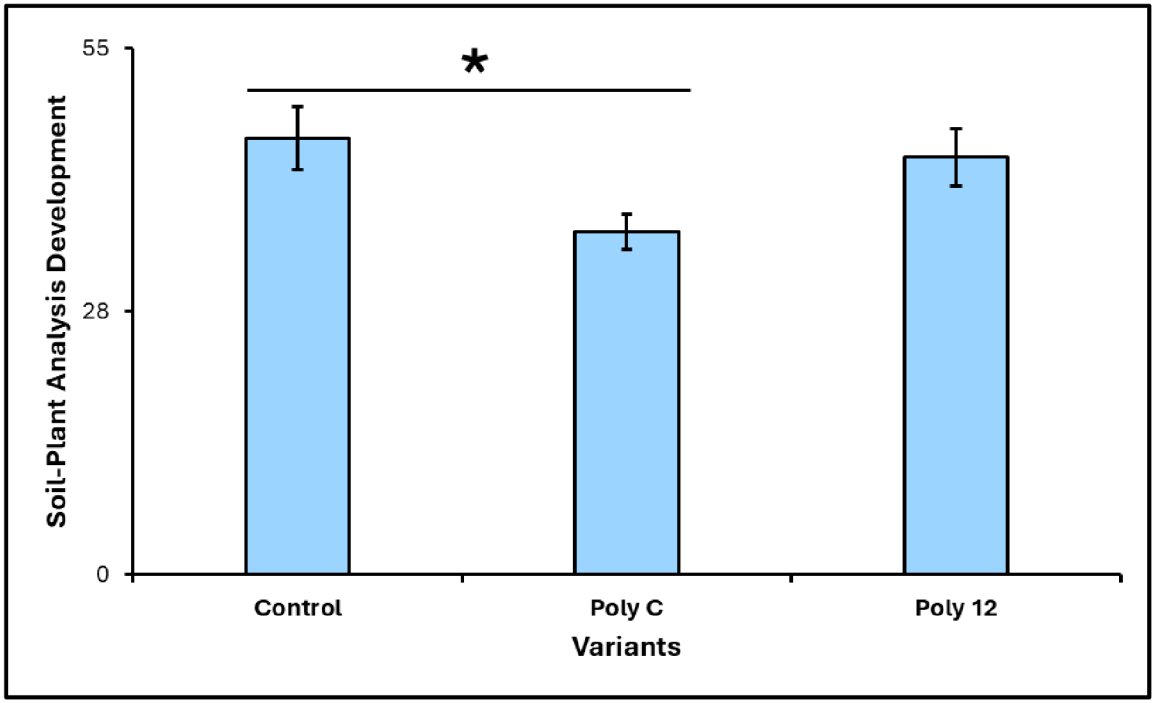

Figure 12: Comparison between the control and autopolyploid genotypes based on the SPAD values. The "*" indicates significant at 5% significance level (compared to the control). The sample size (n) is 10.

4.4 *Terpene synthase* and *Cinnamate 4-hydroxylase* gene expression dynamics: control vs polyploid

The results for the RNA extraction and the PCR (with the gene-specific primers) can be found in the figure 13. In the case of *terpene synthase*, both the polyploid genotypes (Poly C and Poly 12) show heightened expression levels compared to the control. Poly C exhibited nearly a 2-fold increase, while the Poly 12 has a slight increase of about 1.25 times compared to the control. In the case of *cinnamate 4-hydroxylase,* Poly C has a moderate increase in the expression level (1.64 times) compared to the control. However, the Poly 12 showed a significant increase in the *cinnamate 4-hydroxylase* gene expression (7.28 times compared to the control), which is notably higher than the other conditions. The details of the melting curve analysis and gene expression results can be found in the figure 14 and 15, respectively.

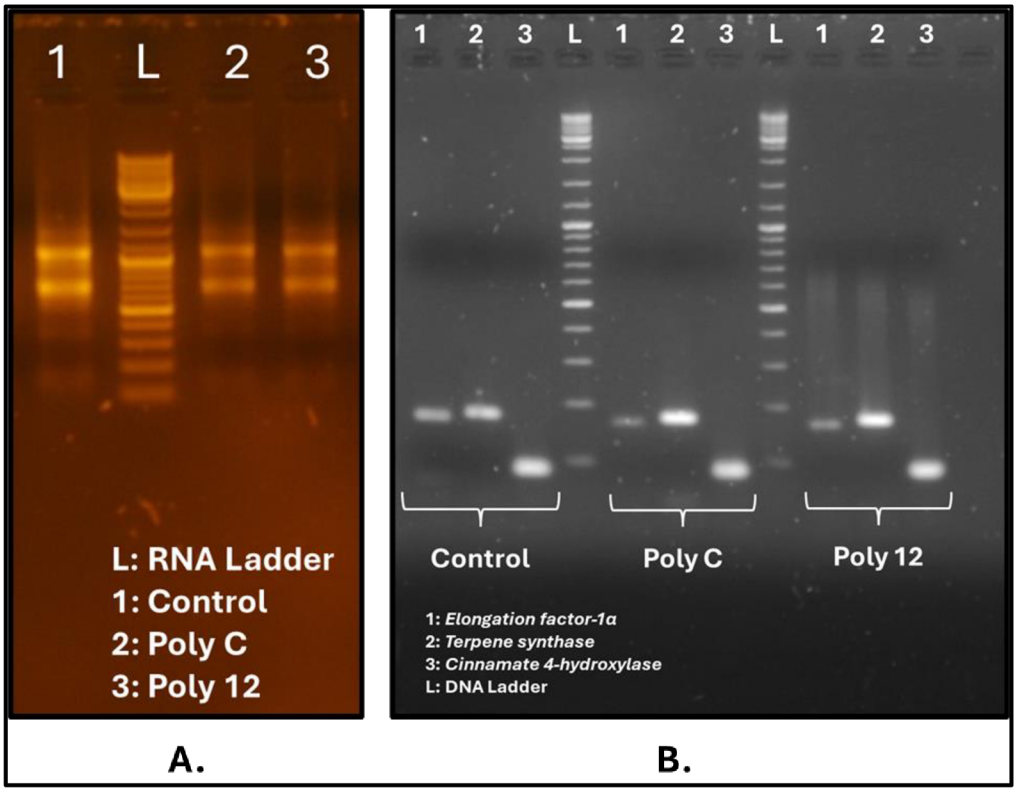

Figure 13: Results for the **(A)** RNA extraction and **(B)** Polymerase chain reaction with the gene-specific primers for the control and the two polyploid plants (Poly C and Poly 12). In the case of RNA, the samples were run in 1.2% agarose gel and the PCR amplicons were ran in 1.8% agarose gel.

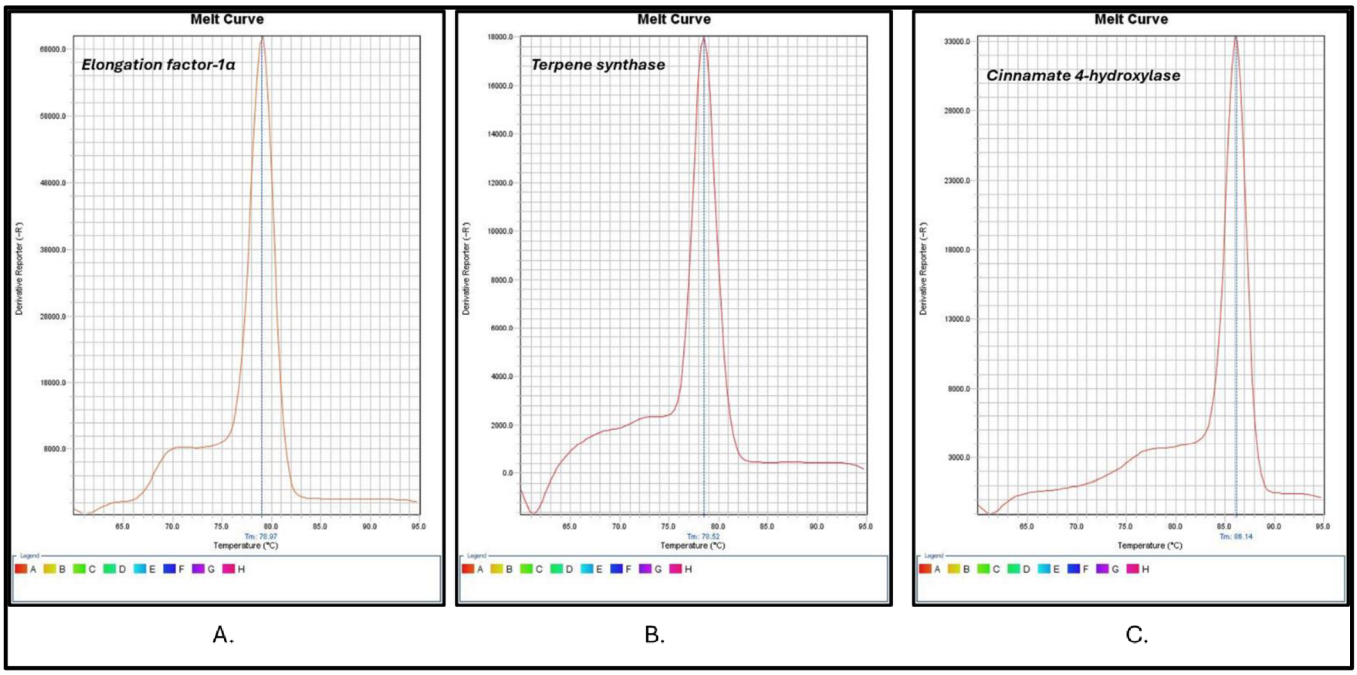

Figure 14: Melt curve analyses of the three genes (A) *Elongation factor 1-α*, (B) *Terpene synthase* and (C) *Cinnamate 4-hydroxylase*. Single melting peaks indicate that there are no non-specific bindings of the chosen primers.

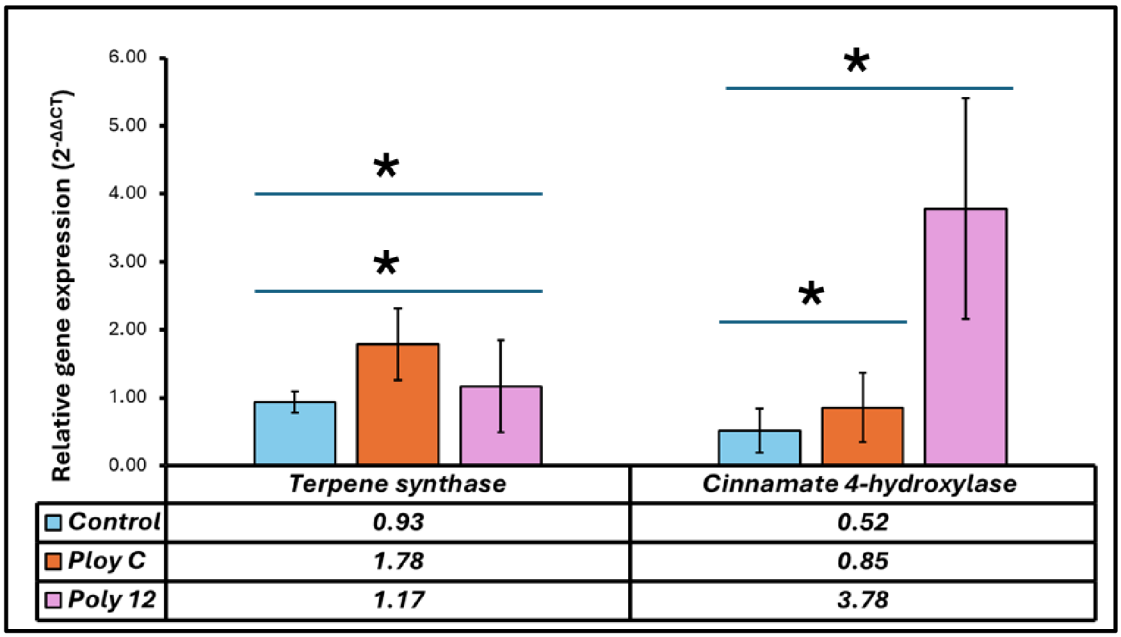

Figure 15: Relative expression level of *Terpene synthase* and *Cinnamate 4-hydroxylase*. The "*" indicates significant at 5% significance level (compared to the control). The sample size (n) is 5.

5. Discussion

The current study on *O. vulgare* and its response to oryzalin treatment provides valuable insights into the survival, polyploid induction, and subsequent phenotypic and genetic changes in this medicinal plant. The first aim of this study was to create autopolyploid *O. vulgare* using oryzalin treatment. To achieve this, we had conducted six different treatments on a total of 312 plants. Our endeavors resulted in two autopolyploid variants (Poly C and Poly 12). Till date, to the best of our knowledge, there are no reports on successful creation of polyploid oregano varieties using oryzalin treatment (under *in vitro* conditions). Hence, the current research marks the first endeavor of its kind, addressing this significant gap in the existing literature. However, the success rate is extremely low, which indicates the challenges and complexities associated with inducing polyploidization in *O. vulgare* using oryzalin treatment under *in vitro* conditions. Moreover, the survival rate showed a consistent decrease with increasing oryzalin concentration and treatment durations. This clearly suggests that while oryzalin can successfully induce autopolyploidy, the process is not easy and may require further optimization for better success rates. While we faced challenges in achieving polyploidization in oregano with oryzalin treatment, it's also worth noting that oryzalin's effectiveness as an antimitotic agent is well-documented across a wide range of plant species. Oryzalin has shown its potential to induce polyploidy in various other plant species. For example, 7 polyploid and 6 mixoploid plant genotypes were obtained in *M. spicata* (Bharati et al. 2023a). In another experiment with watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), the authors obtained 84% polyploidy rate with 25 mg L^{-1} oryzalin concentration. The authors used six different concentrations of oryzalin (0, 5, 10, 15, 20, and 25 mg L^{-1}). Polyploidy was not induced at the lower concentrations (0 - 10 mg L^{-1}). However, at 15 mg L^{-1} and 20 mg L-1, mixoploidy was found. At 25 mg L^{-1}, 84% of the plants exhibited tetraploidy (Bae et al. 2020). In another study with *Capsicum frutescens* L., the authors found 40% polyploidy induction rate with 20 mg L^{-1} and 30 mg L^{-1} oryzalin concentrations. Similar results were also found in *Watsonia lepida* (Ascough et al. 2008) and *Passiflora edulis* Sims. (Rêgo et al. 2011), where oryzalin was successfully in inducing polyploidy.

Our second aim was to explore the effects of induced polyploidization on *O. vulgare*. We assessed the parameters such as number of shoots, shoot length, number of nodes per shoot, internodal distance, number of leaves per shoot, leaf area and thickness, stem thickness, etc. The observed increase in leaf size in the autopolyploid plants compared to the control might indicate

that the autopolyploid plants might have better efficiencies in capturing and utilizing different resources, such as light, water, and nutrients. Moreover, the larger leaf surface area might also allow the polyploid plants to capture more sunlight for photosynthesis and absorb more water and nutrients from the soil, which ultimately might boost their overall growth and development. No statistical differences were found in the number of shoots between the control and the autopolyploids. However, the polyploid plants exhibited significant differences in the shoot length compared to the control. This clearly suggests that polyploidization might influence shoot elongation more than shoot initiation or proliferation in this study. The leaf thickness between the control and polyploid plants showed significant differences, suggesting that polyploidy influenced the leaf morphology. This could be related to changes in cell size or cell number in the leaf tissues, which might have occurred due to polyploidization. In terms of stem thickness, only Poly 12 plants displayed a significant difference compared to the control. The possible explanation for this might be associated with enhanced vascular tissue development or changes in secondary growth processes influenced by polyploidy. In a previous study conducted by Corneillie et al (2019), generated *Arabidopsis* plants with varying somatic ploidy levels (2n, 4n, 6n, and 8n) and conducted detailed phenotypic analyses. They found that the polyploids grew slower than diploids, but the cell size increased and cell number per leaf blade reduced with increasing ploidy levels. Additionally, they also found that that lignin and cellulose content decreased with increasing ploidy. However, the matrix polysaccharide content and the saccharification yield increased (Corneillie et al. 2019). Similarly, in a study with polyploid *Thymus persicus*, the authors found that tetraploid *T. persicus* displayed lower plantlet height, shorter roots, thicker stems, and darker leaves. In addition to thus the tetraploid plants also showed longer and wider stomata and reduced stomatal density on the abaxial and adaxial leaf surfaces (Tavan et al. 2015). Analogously, in our study, we observed significant differences in stomatal size and frequency between the control and autopolyploid plants. In this case, even though the autopolyploid plants demonstrated reduced stomatal numbers but there was an increase in both stomatal length and width. The Poly 12 genotype exhibited a notably longer stomatal length compared to the control, whereas Poly C showed a modest increase (but not statistically significant). Regarding the stomatal width, both autopolyploid genotypes displayed wider stomata compared to the control. These results show that the polyploidization can potentially affect the plant's gas exchange efficiency. Additionally, the larger stomata might also contribute to reduced stomatal density, which might be linked with decreased transpiration rates. This is an

important observation because polyploid plants might have better water-conserving efficiency in challenging environmental conditions. The longer stomatal length and wider stomatal width could be adaptive responses to these challenges. In a very recent study, a group of researchers explored stomata variation during the process of polyploidization in *Allium tuberosum*. The authors concluded that there might be a complex relationship between stomata morphology and polyploidization (Yao et al. 2023). In another comparative study on leaf stomata profiles among different ploidy levels of bananas (*Musa* L.), the authors found distinctive patterns in the stomatal characteristics. The authors concluded that ploidy level have an inverse correlation with the number of stomata but a positive correlation with stomatal length and size. The diploid plants had a higher number of stomata, but smaller stomatal length and size compared to triploids (Auliya et al. 2019).

Apart from the comparison based on the morphological characteristics, we had also analysed the various physiological parameters provided such as gH+, LEF, SPAD, NPQt, Fv/Fm and Pi_Abs. The gH+ value is a measurement of the hydrogen ion conductance or proton flux across the membrane. This parameter denotes membrane integrity and function. In our study, we found that the poly C has the highest permeability. This clearly suggests that polyploidization induces alterations in membrane composition or structure. This might also influence the overall plant health and stress response mechanisms. LEF value indicates the effectiveness of energy flow in the photosynthetic system. Marginally higher LEF in Poly 12 indicates a potentially more effective energy utilization in its photosynthetic pathways. The SPAD value is a measurement of the chlorophyll content. A higher SPAD value implies increased chlorophyll content. In our case, while the variations in SPAD values across the variants suggest differences in photosynthetic vigor, the consistent Fv/Fm values indicate that the polyploidization did not adversely affect the maximum quantum yield of Photosystem II. Hence, we can conclude that despite differences in chlorophyll content, the fundamental efficiency of the photosynthetic process remained stable. Thus, polyploidization has no adverse effects on the photosynthetic efficiency. Contrary to our results, a study with tetraploid *Lilium regale* have shown abnormal chloroplast structure, reduced photosynthetic pigments, and lower photosynthetic rates compared to their diploid counterparts (Wang et al. 2021). This difference underlines the fact that there might be variability in responses to polyploidization across different plant species. Hence, here we also recommend performing species-specific evaluations while assessing the effects of polyploidization on plant physiology, especially photosynthetic efficiency. In addition to these parameters, we had also evaluated the

phosphate absorption capacity and photoprotection ability. The higher Pi_Abs observed in Poly 12 specifies a potential advantage in phosphate uptake and utilization. This factor is considered essential for plant growth and development, especially under nutrient-limiting conditions. Concurrently, the elevated NPQt in Poly C suggests an adaptive mechanism to dissipate excess light energy as heat. These individual physiological responses in Poly 12 and Poly C underscore their unique adaptations to nutrient availability and environmental conditions, emphasizing the intricacies of polyploid plant responses.

The heat-map from the cluster analysis (Figure 16), utilizing eight comparable parameters including number of shoots, shoot length, leaf width, leaf length, leaf thickness, stem thickness, stomata length, and stomata width), distinctly highlighted the differences between the control and its autopolyploid variants. Among the autopolyploids, Poly C exhibits morphological traits more similar to the control than Poly 12. Notably, the most distinctive trait observed is the stem thickness.

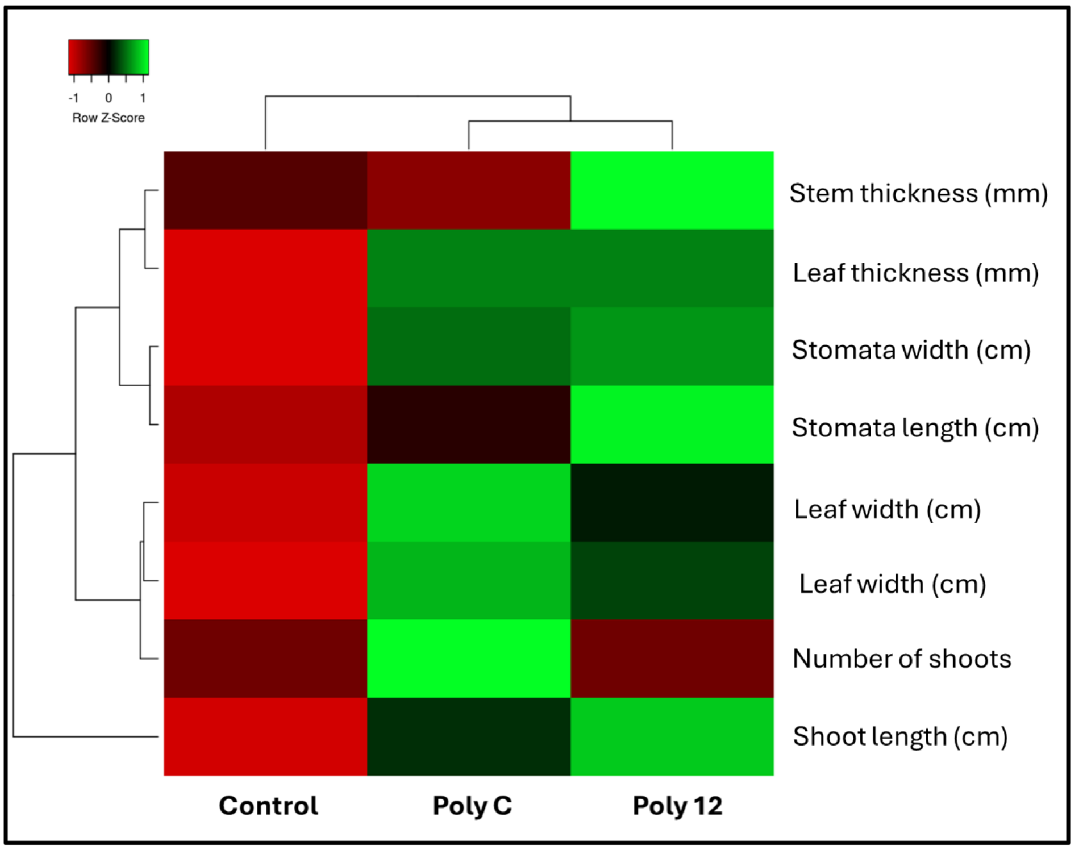

Figure 16: Heat-map from the cluster analysis. The heat-map was constructed using http://heatmapper.ca

Our third aim was to explore the effects of induced polyploidization on the gene expression patterns of *terpene synthase* and *cinnamate 4-hydroxylase*. In *O. vulgare*, its aroma, flavor, and pharmaceutical values are accredited to its essential oil, which primarily consists of monoterpenes and sesquiterpenes. Terpene synthases play vital roles in the oxidation and cyclization of precursors involved in the production of monoterpenes and sesquiterpenes (Crocoll et al. 2010). In the current study, both autopolyploid genotypes showed increased *terpene synthase* expression. This indicates that induced polyploidization positively influences *terpene synthase* expression, potentially enhancing terpene-related functions in these genotypes. Cinnamate 4-hydroxylase is a cytochrome P450 monooxygenase found on the outer surface of the endoplasmic reticulum in plants. This enzyme catalyzes the conversion of cinnamic acid into 4-coumaric acid and hence plays vital roles in the phenylpropanoid metabolic pathway (Khatri et al. 2023). In this study, both polyploid genotypes exhibited higher expression of *cinnamate 4-hydroxylase*. This suggests that induced polyploidization has a positive effect on phenylpropanoid metabolic pathway. Similar to our study, previous studies had also assessed the expression of genes related to terpene biosynthesis and phenylpropanoid metabolic pathways. For example, in a comparative study on the production of bioactive secondary metabolites in diploid and tetraploid *Echinacea purpurea* (L.) Moench, the authors discovered that tetraploidization boosted the activity of important metabolic pathway enzymes including cinnamate 4-hydroxylase (Xu et al. 2013). However, contrasting results were obtained in *Solanum bulbocastanum*, where the tetraploids have lower levels of phenylpropanoids, compared to diploids (Caruso et al. 2013). On the other hand, the results indicated that the phenylpropanoid content was generally higher in the tetraploid *Solanum commersonii* Dun. compared to the diploid form (Caruso et al. 2011). This clearly suggests that chromosome doubling doesn't unanimously enhance the production of bioactive compounds across all the plant species.

Our fourth aim was to provide insights into the practical implications for the agricultural and medicinal applications of oregano based on our study's findings. Based on the comprehensive findings of our study the possible practical implications for agricultural and medicinal applications are as follow:

- **Agricultural implications**
  - **A.** Our study can be considered as an innovative effort in successfully inducing polyploidization in *O. vulgare* using oryzalin treatment. Even though the polyploidy induction success rate is at present low, additional optimization may possibly increase the

efficiency of polyploid induction. Moreover, the creation of polyploid varieties could lead to novel cultivars with better-quality traits. This might contribute to the overall robustness under unpredictable environmental conditions.

B. Secondly, we observed larger stomata in polyploid oregano plants. This might be an adaptive response to environmental challenges and cultivating these plants might be beneficial for drought-prone regions. Hence, polyploid oregano varieties might play a role in sustainable agriculture by reducing water usage.

C. In addition to the above-mentioned points, we had also observed an increased phosphate absorption capacity in the polyploid genotypes. Hence, these plants might have an advantage while growing in nutrient-poor soils. This could be remarkably useful for growing under agricultural settings with limited nutrient availability.

- **Medicinal Implications**

A. Oregano's medicinal properties are largely accredited to its essential oil content, which predominantly consists of monoterpenes and sesquiterpenes. We observed an increased expression of *terpene synthase* in polyploid oregano genotypes. This clearly suggests that these plants might have better capacity to produce essential oils. However, this finding requires additional experiments with the aim to identify the essential oil composition. Subsequently, this might possibly lead to the development of oregano varieties with higher medicinal value.

B. The higher expression of *cinnamate 4-hydroxylase* in polyploid oregano genotypes also indicates a positive influence on the phenylpropanoid metabolic pathway, an extremely important pathway leading to the production of a variety of secondary metabolites with probable medicinal properties. Therefore, the polyploid oregano plants might also be cultivated for improved bioactive compound production, which could be of interest for medicinal applications.

Finally, the future research directions might include employing functional genomics-based studies, including investigations based on broader transcriptomic and metabolomic changes associated with polyploidization. This will help us to discover the key genes and pathways involved in the response to polyploidization and the biosynthesis of bioactive compounds. While our current study undoubtedly offers valuable insights into its agricultural and medicinal applications, it's also essential to acknowledge its potential drawbacks. The potential drawbacks include:

A. The first and foremost challenge in *in vitro* polyploidization studies is the time constraint. Extended research periods could provide a broader understanding.

B. We obtained a very low number of polyploid varieties. This clearly suggests that inducing polyploidization with oryzalin in *O. vulgare* can be challenging and complex. Hence, we recommend further optimization of the oryzalin concentrations and time durations for better success rates.

C. Considering the wide-range of effects of polyploidization, our results might not be generalized to all oregano varieties or growing conditions. Hence, we recommend conducting future experiments using a broader range of samples. Increasing the sample size and diversity might provide more comprehensive insights into this area of research.

D. Due to lack of time and considering the fact that the success rate is not high, we could not study the possible effects of environmental conditions on the plant's growth and chemical composition. Hence, the current results might be/not be affected by factors like temperature, rainfall, and soil quality. Hence, we recommend replicating the findings in different settings or seasons.

E. We chose the reference gene based on previous literature. But it is always recommended to consider experiment specific reference genes. Hence, we also recommend considering pilot experiments on choosing the most relevant reference gene, prior to replicating the gene expression studies.

F. Another drawback is that the current study have not compared oregano with other plants or treatments. Hence its relative efficacy is still a huge research gap.

G. Last, but not the least, due to time constraints, we have not focused on the performance of the obtained polyploid genotypes under specific geographical regions. Hence, we have neglected the global variations parameter, thus limiting the study's global applicability and relevance.

6. Conclusions

The current study aimed to explore the effects of oryzalin-induced polyploidization on *O. vulgare* L., a versatile medicinal plant from the Lamiaceae (or mint) family. Most importantly, the current thesis reports the first successful creation of autopolyploid oregano varieties using oryzalin treatment (under *in vitro* conditions). Additionally, this study have successfully achieved the aims and hypotheses set forth in this study. The current study had obtained 11 autopolyploid variants from 312 plants (6 different treatments). The flow cytometry confirmed doubled DNA content in these autopolyploid samples (Poly C and Poly 12), despite a low autopolyploidization rate, marking a significant milestone in successful polyploid oregano creation. Additionally, the autopolyploid plants exhibited distinct growth characteristics compared to the control, with larger leaves, longer shoots, and varied leaf and stem thickness. Autopolyploidization also influenced stomatal size and frequencies. Interestingly, no significant differences in the photosynthetic efficiency (Fv/Fm) were obtained. However, the variations in the SPAD values suggest differences in chlorophyll content. Overall, these results indicate that autopolyploidization does not have detrimental effects on the photosynthesis however there might be influences on the photoprotection mechanisms.

In summary, the current study provides valuable insights into the effects of oryzalin-induced autopolyploidization on *O. vulgare*. The successful creation of polyploid oregano will mark a significant advancement in plant polyploidization research. However, added researches are needed to explore the long-term effects of polyploidization and its practical implications for agricultural and medicinal applications. These researches might include assessment of the genomic stability of the polyploids, conducting transcriptomic studies to understand global gene expression changes in the polyploid plants and examining the secondary metabolite profiles of polyploid plants to assess potential changes in medicinal compound production.

7. References

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