Czech University of Life Sciences Prague

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Exploring the Potential of *In-vitro* Polyploidization for Genetic Improvement of Medicinal Plants

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Declaration

I affirm and attest that the contents presented within this thesis, titled 'Exploring the potential of *in-vitro* polyploidization for genetic improvement of medicinal plants,' and submitted as a partial fulfillment of the requirements for the Ph.D. at the Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague, is my own work, unless explicitly acknowledged in the references or acknowledgments sections. Additionally, I affirm that none of the materials presented in this work have been submitted for any other academic degree, either within this university or any other institution.

Prague, 24th April 2024

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Rohit Bharati

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List of Figures

List of Tables

Table of Contents

List of abbreviations

APM	Amiprophos-methyl
CZU	Czech University of Life Sciences
DAD	Diode array detector
DAPI	4′,6-diamidino-2-phenylindole
DH	Doubled haploid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
FCM	Flow cytometry
FID	Flame ionization detector
FTA	Faculty of Tropical AgriSciences
GABA-T	Gamma-aminobutyric acid transaminase
GC/MS	Gas chromatography-mass spectrometry
GS	Genomic selection
GWAS	Genome-wide association studies
HCl	Hydrochloric acid
HPLC-DAD	High-performance liquid chromatography with diode-array detection
ICP-OES	Inductively coupled plasma - optical emission spectrometry
MAS	Marker assisted selection
M-phase	Mitotic phase
mRNA	Messenger ribonucleic acid
MS	Murashige and skoog (1962) medium
PCR	Polymerase chain reaction
PTFE	Polytetrafluoroethylene
QTL	Quantitative trait loci
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
rpm	Revolutions per minute
SNP	Single nucleotide polymorphism
SPAD	Soil plant analysis development
TE	Testing population
TR	Training population
UPGMA	Unweighted pair group method with arithmetic mean

Abstract

Medicinal plants have been integral to human survival since ancient times, serving diverse purposes ranging from pharmaceutical applications to the production of perfumes, cosmetics, and flavoring agents, etc. Traditional breeding approaches have been employed but are hampered by time and capital constraints, taking up to a decade to create novel cultivars. Consequently, minor crops, including important medicinal plants, receive inadequate attention from breeders and researchers. To address these challenges, the study explores synthetic polyploidization, a cost-effective and time-efficient method that involves chromosome doubling to generate novel genotypes with enhanced traits. The thesis focuses on the genetic improvement of medicinal plants such as spearmint (*Mentha spicata* L.), lemon balm (*Melissa officinalis* L.), basket plant (*Callisia fragrans* (Lindl.) Woodson), and mini melon (*Melothria scabra* Naudin) through *in vitro* polyploidization. Additionally, the thesis also explores the application of this method in *Vitis* spp., while addressing potential challenges associated with chromosome doubling. The *in vitro* polyploidization of the selected plants involved treating the nodal segments of the plants with varying concentrations (1-100 μM) and duration (24h & 48h) of oryzalin. Thereafter, polyploid plants were screened among the survived treated plants using flow cytometry or chromosome counting or both. The findings demonstrate that oryzalin is an effective antimitotic agent in inducing autopolyploid in the studied plants where the polyploid induction rate ranged between 8-15%. The induced polyploids displayed significant variations compared to their progenitor genotypes in terms of morphological, biochemical, biological, anatomical, and physiological characteristics. These findings underscore the transformative impact of synthetic polyploidization on the genetic enhancement of medicinal plants, offering a promising avenue for accelerated breeding and the development of cultivars with improved therapeutic properties. This comprehensive exploration contributes valuable insights to the field, bridging the gap between traditional breeding methods and innovative approaches for sustainable medicinal plant cultivation and utilization.

Keywords: crop improvement, *in vitro*, medicinal plants, oryzalin, polyploidization

Abstrakt:

Léčivé rostliny tvořily významnou část lidského jídelníčku již od starověku a byly využívány k mnoha dalším účelům, např. výrobě parfémů, kosmetiky a jako ochucovadla. Metody tradičního šlechtění byly sice využívány, ale ukázalo se, že vzhledem k jejich časové a finanční náročnosti, je potřeba vyvíjet nové kultivary pomocí moderních přístupů. V určitých historických obdobích však minoritní plodiny, včetně léčivých rostlin, byly z různých důvodů ve šlechtitelských a výzkumných projektech opomíjeny. V poslední době se opět léčivé rostliny dostávají do popředí zájmu, a proto bylo nutné vyvíjet nové kultivary. Pro jejich šlechtění se využívají metody umělé polyploidizace, metody časově i finančně náročné, která vychází z předpokladu, že zvýšením počtu chromozomů dojde k významnému zlepšení některých znaků u takto vzniklé polyploidní rostliny. V této disertační práci byly studovány čtyři různé léčivé rostliny, Mentha spicata L., Melissa officinalis L., Callisia fragrans (Lindl.) Woodson a Melothria scabra Naudin pomocí in vitro polyploidizace. Pro ověření postupů byla dále tato metoda testována na druhu Vitis spp. In vitro polyploidizace u vybraných rostlin zahrnovala ošetření nodálních částí rostlin odlišnými koncentracemi oryzalinu (1-100 μM), zároveň se lišila délka expozice této látce (24h & 48h). Poté, byl u polyploidních rostlin, které ošetření přežily, stanoven počet chromozomů pomocí průtokové cytometrie nebo klasických karyologických metod (případně kombinací obou metod). Ukazuje se, že orylazin je vhodným agens vykazující antimikrotubulární aktivitu, které lze využít k indukci autopolyploidizace u studovaných rostlin. U indukovaných rostlin se podíl polyploidních jedinců pohyboval v rozpětí od 8 do 15%. Takto vzniklé polyploidní rostliny vykazovaly změny v morfologických a anatomických znacích, biochemických a fyziologických charakteristikách. Výsledky této práce představují významný krok směrem k vývoji nových kultivarů léčivých rostlin s lepšími léčivými vlastnostmi pomocí moderních přístupů. Závěrem lze konstatovat, že výsledky této práce přispěly do značné míry k vyplnění určité mezery mezi tradičními šlechtitelskými přístupy a inovativními, jejichž použití bylo u léčivých rostlin úspěšně vyzkoušeno.

Kličova slova: šlechtění rostlin, *in vitro*, léčivé rostliny, oryzalin, polyploidizace

1. Introduction

Medicinal and aromatic plants have been used to fulfill human requirements from time immemorial. These medicinal plants possess crucial metabolites having numerous applications across different industries. Primarily, these plants act as source of metabolites with pharmaceutical potential. For the same reason, nearly 80% of the synthetic drugs are directly or indirectly derived from the medicinal plants (Bauer and Brönstrup, 2014). While synthetic drugs are predominantly used in the western world, according to the World Health Organization (WHO), 80% of the population from developing nations still benefits from the traditional medicine obtained from the medicinal plants (Kumar et al., 2011; Vaou et al., 2021). For example, willow bark (genus Salix) produces salicylic acid which is a precursor used for the synthesis of Aspirin, a common drug used for relieving pain and fever (Vlachojannis et al., 2011). Taxol is another excellent example, where it is used for its anti-cancer properties and is extracted from the Pacific yew tree (*Taxus brevifolia*) (Li et al., 2014). Apart from medicinal and ethnopharmacological properties, medicinal plants are extensively used for the production of perfumes, cosmetics, flavoring agent and ornamental purposes.

As medicinal plants are of immense importance to human survival, continuous breeding of these plants to generate novels genotypes with enhanced therapeutic compounds and activities is imperative. Traditionally, several breeding approaches including mass selection, pure line selection, hybridization, backcrossing, inbreeding, etc., have been employed for genetic improvements. However, these traditional methods have serious challenges and limitations. The traditional methods are often time and capital intensive. As the traditional methods involve various phases, it could roughly take 8 – 10 years or even more to create novel cultivar or genotype (Temesgen, 2022). For the same reason, minor crops and other important plants like medicinal plants get less attention from the breeders and researchers. However, there are other economically feasible and faster approaches that these medicinal plants could benefit from. One such method is synthetic or artificial polyploidization, also known as chromosome doubling, that could generate novel genotypes with enhanced traits in significantly less time and capital (Niazian and Nalousi, 2020). Apart from being cost effective and time efficient, synthetic polyploidization holds immense potential to enhance morphological, physiological and biochemical characteristics of medicinal plants.

Artificial polyploidization can exert a wide range of effects on the traits of plants. The quite apparent change in the induced polyploid is the morphological variation in the plant organs

including leaves, flowers, stems, roots, fruits, etc. (Madani et al., 2021; Rauf et al., 2021). For example, artificial polyploid induced increased leaf length, plant height, inflorescence length and thicker stems in *Trachyspermum ammi* L. (Sadat Noori et al., 2017). Similarly, leaf length, leaf width and the number of leaves increased in the induced tetraploid plants of *Paphiopedilum villosum* (Huy et al., 2019). Apart from morphology, induced polyploid plants also possess altered anatomical characteristics including larger cells, stomata, and trichomes (Tavan et al., 2015; Talebi et al., 2017; Zhou et al., 2020; Gantait and Mukherjee, 2021). Studies have also been successful in obtaining polyploid plants with enhanced secondary metabolites, highlighting the potential of genome duplication in modulating gene expression resulting in higher secondary metabolites. Some examples include enhanced essential oil yield in mojito mint (*Mentha* × *villosa*), *Lippia integrifolia* and *Zingiber officinale* Roscoe (Iannicelli et al., 2016; Moetamedipoor et al., 2022; Prasath et al., 2022).

Considering these developments and outcomes, the thesis explores the potential of synthetic polyploidization in genetic improvement of different plants with medicinal and therapeutic properties, namely, spearmint (*Mentha spicata* L.), lemon Balm (*Melissa officinalis* L.), basket plant (*Callisia fragrans* (Lindl.) Woodson), mini melon (*Melothria scabra* Naudin). The thesis also explores the perspective of chromosome doubling in *Vitis* spp and the potential challenges associated.

2. Literature Review

2.1 Plant polyploidy

Plant polyploidy is the presence of more than two sets of chromosomes in a cell. It plays a pivotal role in the evolutionary dynamics and genetic diversity of plant species. Polyploids are quite common among plants, particularly angiosperms along with a few groups of amphibians and fish (Woodhuse et al., 2009). Through the course of evolution plants have utilized polyploidy to adapt and survive in diverse and harsh environmental conditions. Studies estimated that nearly 30-70% angiosperms have gone through at least one genome duplication (Sattler et al., 2016). With advancement with genomics data availability, it is becoming evident that plants have gone through repeated genome duplication events throughout evolutionary history (Panchy et al., 2016).

Polyploidy is generally of two types: autopolyploidy and allopolyploidy (Leitch and Bennett, 1997). Autopolyploidy arises within a single species, typically through chromosome duplication events within the same genome, resulting in individuals with multiple sets of chromosomes derived from their own species. Additionally, autopolyploids are homozygous at each locus in the genome. In contrast, allopolyploidy involves the fusion of different but related species, producing hybrids with mismatched chromosome numbers. To overcome this disparity and restore fertility, these hybrids undergo chromosome doubling, yielding organisms with complete sets of chromosomes from each parent species. Both autopolyploids and allopolyploids play significant roles in evolution, contributing to biodiversity, enhanced adaptability, and, in the case of plants, often leading to increased vigor and economic relevance in agriculture (Van De Peer, 2023) (Figure 2.1).

Polyploid plants are observed to have superior traits compared to their diploid counterparts, giving them survival advantages. The most common trait is the heterosis or the hybrid vigor observed in the polyploid offspring obtained from two diploid parents. One of the primary reasons behind this observed trait is the prevention of recombination. In polyploid organisms, especially allotetraploids, the chromosomes from the original parents are forced to pair up. This pairing prevents recombination between the genomes of the original parents thus maintaining heterozygosity (Woodhuse et al., 2009). Another reason is attributed to gene redundancy, that provides the polyploid plants with twice the number of genes for a trait. It also protects the polyploids from the deleterious effects of recessive mutations during the gametophyte life stage protecting the pollen or egg sac from developmental dysfunction (Woodhuse et al., 2009).

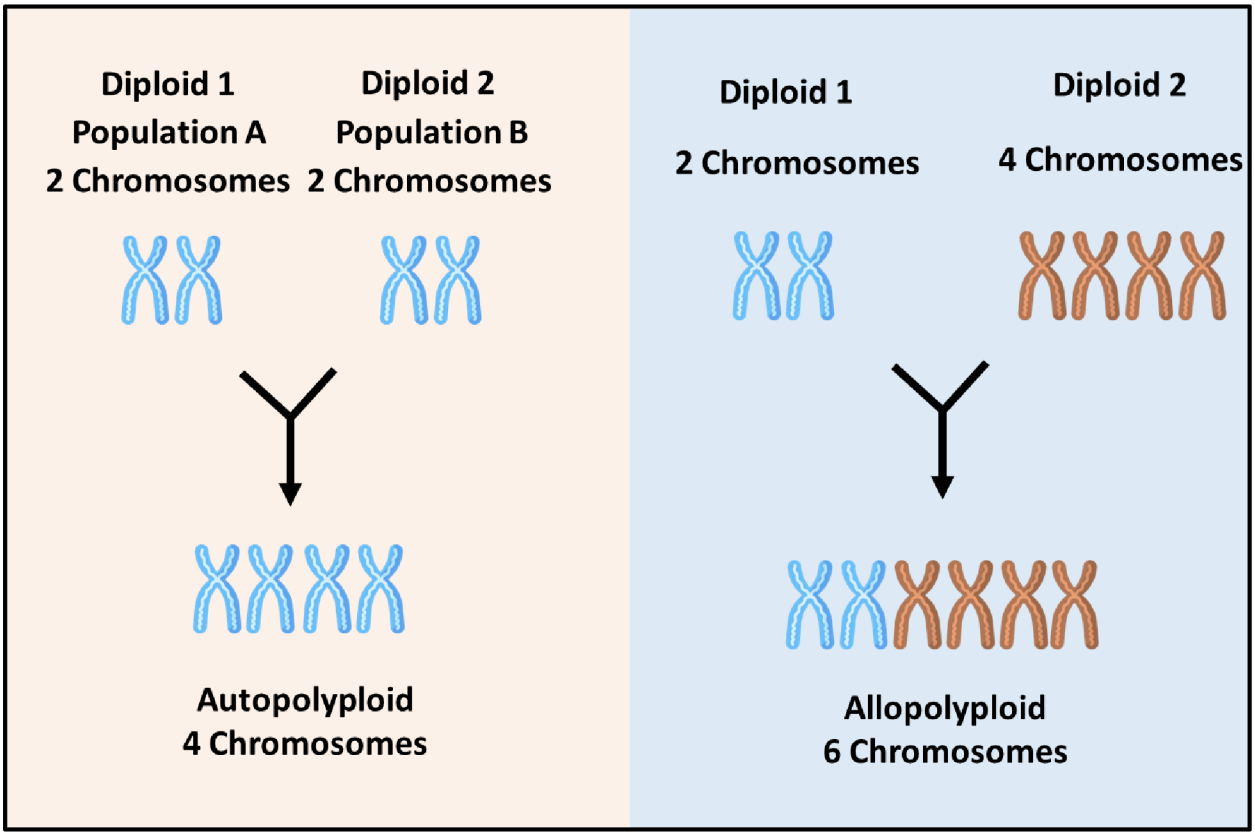

Figure 2.1 Simplified mechanism involved in the formation of autopolyploid and allopolyploid. Image source: Author

2.2 Artificial Polyploidization

Artificial polyploidization is a deliberate and controlled process employed in plant breeding to induce genome duplication. This intentional manipulation of the plant genome allows for the development of new genotypes with enhanced traits (Niazian and Nalousi, 2020). The exploration of artificial polyploidization techniques opens avenues for improving various aspects of plant biology, including medicinal properties. Induced polyploids can be generated through physical method or chemical methods. The physical methods include temperature shock, radiation, grafting, etc. Physical methods have been used to induce polyploidy in some plant species, however, due to its low efficacy it is not widely used. Chemical methods that involve treatment of chemical antimitotic agents like colchicine to the plants are more frequently used due to its high effectiveness, lower cost, and minimal expertise requirements (Chen et al., 2021).

2.2.1 Working principle of artificial polyploidization

Artificial polyploidization relies on the manipulation of the plant's cell cycle to induce polyploidy. The plant cell cycle progresses through various phases, such as G1-phase, S-phase, G2-phase, and M-phase (mitosis) (Francis, 2007). The crucial aspect of inducing polyploidization involves disrupting the cell cycle between the S-phase and the completion of mitosis, resulting in the cell possessing double amount of nucleic acid content (Dhooghe et al., 2011). Chemical compounds that interfere with the cell cycle during the later stages of the M-phase are considered viable options for chemically induced polyploidization. Metaphase inhibitors, a notable group of antimitotic agents, play a pivotal role in this procedure. These inhibitors disturb the metaphase by binding with α and β-tubulin dimers, essential components of the microtubule spindle fibers, crucial for accurate polar migration of chromosomes (Dewitte and Murray, 2003). Consequently, the separation of chromosomes is hindered, resulting in cells with a doubled number of chromosomes. The intricate manipulation of the cell cycle and the precise disruption of crucial molecular processes define the underlying principles of artificial polyploidization.

2.2.2 Antimitotic agents

Chemical anti-mitotic agents play a crucial role in the success of artificial polyploidization. Several commonly used antimitotic agents are employed to disrupt cell division and promote the formation of polyploid cells. While there are numerous mitotic inhibitors available, some of the well-known and employed anti-mitotic agents include colchicine, colcemid, oryzalin, trifluralin, and amiprophos-methyl (Dhooghe et al., 2011). Colchicine is one of the classic examples widely utilized that hinders microtubule formation and, consequently, mitosis (Eng and Ho, 2019). Colchicine is commonly used due to its thermostability and its efficacy over diverse range of plants (Zhang et al., 2007; Fernández-Cusimamani et al., 2023). However, colchicine has numerous side effects including abnormal growth, sterility, gene mutation (Dhooghe et al., 2011). Oryzalin, a dinitroaniline herbicide, serves as an alternative that acts similarly to colchicine by disrupting microtubules but has a higher binding affinity towards plant tubulin dimer compared to the commonly used colchicine (Dhooghe et al., 2011; Beranová et al., 2022). Due to this reason oryzalin often is much more effective at lower concentration compared to colchicine (Bharati et al., 2023a). Overall, these agents are carefully applied during specific stages of *in vitro* tissue culture to achieve the desired ploidy level without compromising the overall health of the cultured tissues. The selection and precise concentration

of these antimitotic agents are critical factors in the success of polyploidization protocols, highlighting their pivotal role in the manipulation of plant genomes for improved traits and genetic studies.

2.2.3 Anti-mitotic application systems

Artificial polyploidization can be carried out under *ex vitro* or *in vitro* conditions. *Ex vitro* conditions include treatment of whole plant or part of plant with a suitable antimitotic agent under experimental setup. The application of anti-mitotic agents in this approach is mainly soaking, immersion, mixed culture, and droplets (Niazian and Nalousi, 2020). This approach has been used to induce polyploids in different plant species including grapevine, common thyme, cumin, cannabis, etc. (Mansouri and Bagheri, 2017; Kara and Doğan, 2023; Mohammadi et al., 2023; Sanaei-Hoveida et al., 2024). Although *ex vitro* treatments have been successful in inducing polyploids in different plants, this approach is quite less effective and yields low frequency of polyploids. In contrast, recent studies have employed *in vitro* applications of anti-mitotic agents to plants providing better control resulting in much higher efficacy (Bharati et al., 2023a). It has also been observed that the anti-mitotic agent treatment under *in vitro* conditions causes significantly less chimerism (Chen et al., 2020).

2.3 *In vitro* polyploidization as a tool for plant trait improvement

In vitro polyploidization has recently become a preferred method to induce polyploidy in economically important plants. It involves treatment of anti-mitotic agent to plants growing under *in vitro* conditions on a predefined plant growth medium for varying concentration and time duration. Depending on the specific anti-mitotic agent and the type of plant, the treatment may involve immersing it in the media along with other salts and hormones, or it can undergo a method where the anti-mitotic solution is directly poured onto the explants placed in the media (Zahumenická et al., 2018; Homaidan Shmeit et al., 2020; Beranová et al., 2022). A general schematic representation of *in vitro* polyploid induction, detection and evaluation using oryzalin as anti-mitotic agent is represented in Figure 2.2.

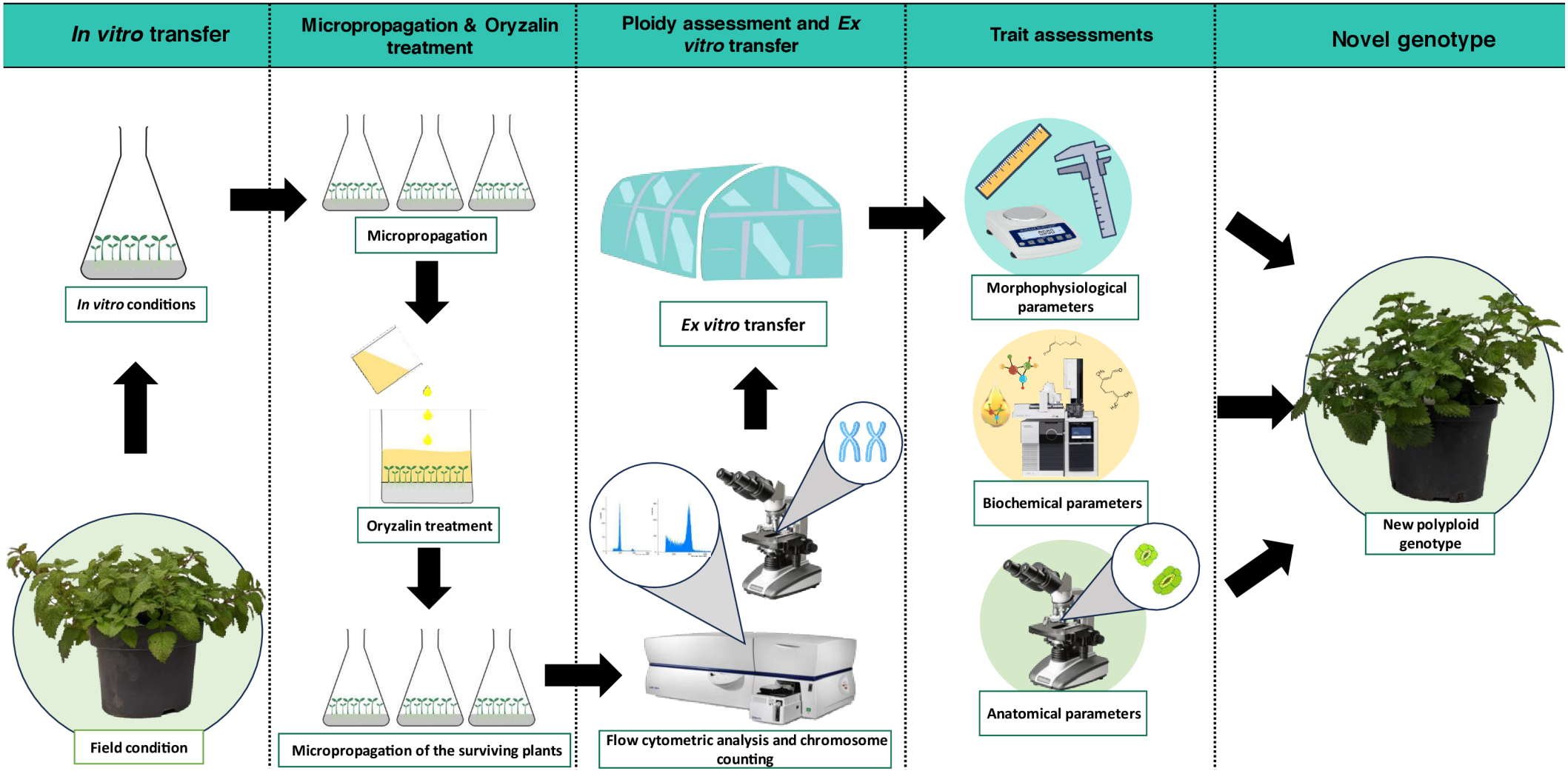

Figure 2.2 A schematic representation of *in vitro* approach for artificial polyploidization using oryzalin as anti-mitotic agent. Image source: Author

2.3.1 Factors influencing *in vitro* polyploidization

The success and efficiency of *in vitro* polyploidization hinge upon a multitude of factors that collectively shape its outcome. Central to this is the *in vitro* regeneration protocol, delineating the procedural steps for tissue culture and subsequent regeneration, exerting a pivotal influence on the induction of polyploidy (Niazian and Nalousi, 2020; Touchell et al., 2020). Other critical factors include the selection of the suitable anti-mitotic inhibitor as well as its concentration and duration, employed to arrest cell division and facilitate polyploid formation (Dhooghe et al., 2011; Touchell et al., 2020). The appropriate selection and calibration of these factors is imperative to attain a higher successful chromosome doubling while preserving the overall health of the cultured tissues. Equally crucial is the careful selection of explants, which can markedly influence the efficiency of polyploid induction (Touchell et al., 2020; Gantait and Mukherjee, 2021). The intrinsic variability in plant genotype across species adds another layer of influence, affecting the responsiveness explants to the polyploid induction process. The physical parameters governing *in vitro* culture, such as temperature, light intensity, and humidity, significantly impact the efficacy of polyploidization (Niazian and Nalousi, 2020). Additional considerations, encompassing the composition of the culture medium, and the overall health of the donor plant material, intricately contribute to the array of elements influencing *in vitro* polyploidization (Niazian and Nalousi, 2020). A comprehensive understanding and optimal manipulation of these factors are imperative for unlocking the full potential of *in vitro* polyploidization in advancing plant improvement and genetic research (Gantait and Mukherjee, 2021).

2.4 Significance of *in vitro* polyploidization in medicinal plants

In vitro polyploidization holds profound significance in the realm of medicinal plants, offering a powerful tool for the enhancement of desirable traits and the improvement of medicinal properties (Salma et al., 2017; Niazian and Nalousi, 2020; Madani et al., 2021; Trojak-Goluch et al., 2021). By inducing polyploidy in medicinal plant species, researchers can modulate the expression of key secondary metabolites, such as alkaloids, flavonoids, and terpenoids, which are often responsible for the therapeutic properties of these plants (Sakhanokho et al., 2009; Iannicelli et al., 2016; Gantait and Mukherjee, 2021). Polyploidization can lead to increased biomass production, altering the quantity and composition of bioactive compounds in medicinal plants (Salma et al., 2017; Neenu et al., 2023). This manipulation offers the potential to amplify the yield of pharmaceutical compounds, contributing to the sustainable production of valuable

drugs. Moreover, *in vitro* polyploidization enables the generation of plants with improved adaptability to diverse environmental conditions, ensuring a more robust and resilient supply of medicinal resources. The controlled nature of *in vitro* polyploidization allows for precision in the breeding process, ensuring the retention of desired medicinal traits. Overall, the application of *in vitro* polyploidization in medicinal plants presents a promising avenue for advancing pharmaceutical research, sustainable resource management, and the development of novel therapeutic agents.

In the current thesis we selected plants with medicinal potential for polyploid induction. The selected plants included spearmint (*Mentha spicata* L.), lemon balm (*Melissa officinalis* L.), basket plant (*Callisia fragrans* (Lindl.) Woodson), and mini melon (*Melothria scabra* Naudin). These plant species were chosen as these plants represent an underexplored niche in medicinal plant breeding efforts. Despite their inherent medicinal potential, these plants have remained relatively overlooked in terms of genetic enhancement. The decision to concentrate on them stems from the anticipation of unlocking their medicinal potential through polyploidization, thereby contributing to the creation of novel genotypes. Furthermore, the choice of these plants is also grounded in their relatively shorter reproductive cycles, facilitating the early expression of the desired traits. This focus on the *in vitro* polyploidization of these medicinal plants aims to unveil their untapped genetic potential, offering insights into sustainable resource management and the development of novel genotypes with enhanced traits. Apart from these plants, thesis also explores a perspective of chromosome doubling in a perennial crop, that are grapevines, and the potential challenges of polyploidization on plants with longer life cycle are discussed.

2.4.1 Spearmint (*Mentha spicata* L.)

Mentha spicata, commonly known as spearmint, is a perennial herb from the Lamiaceae family with an upright growth pattern and rhizomatous roots. It is often cultivated for culinary purposes and as a ground cover. The plant typically reaches heights of 30-100 cm, though it can occasionally grow up to 60 cm. Characterized by variably hairless to hairy stems and foliage, spearmint features wide-spreading fleshy underground rhizomes. Its leaves, measuring 5–9 cm in length and 1.5–3 cm in width, have a serrated margin and emit a strong spearmint fragrance and flavor. The flowering stage of spearmint is marked by slender spikes, with each flower being either pink or white and the inflorescences measuring 3-12 cm long and 5-10 mm wide (Mahendran et al., 2021) (Figure 2.3). Spearmint has a broad range of geographical distribution

Figure 2.3 *Mentha spicata* L. growing in the wild (Image source :https://theoriginalgarden.com).

extending from Ireland in the west to southern China in the east. However, it is native to Africa, Europe and southern temperate regions of Asia (Kumar et al., 2011). This herb has successfully naturalized in various temperate regions worldwide, including northern and southern Africa, North America, and South America. Recognizable by its unique aroma, spearmint is widely introduced across the north temperate zones. Spearmint holds particular significance in these regions, constituting a substantial portion of the overall mint production area.

Spearmint holds a rich history of diverse traditional and ethnopharmacological applications across different cultures. Beyond its role as a culinary herb, spearmint has been utilized for

various ethnopharmacological purposes, including the treatment of conditions such as cold, cough, asthma, fever, obesity, jaundice, digestive problems, sore throat, headaches, toothaches, cramps and inflammation of the respiratory tract (Cakilcioglu and Turkoglu, 2010; Babaeian et al., 2015; Baydoun et al., 2015; Ali-Shtayeh et al., 2019; Mahboubi, 2021). For example, in Iran, aerial parts of the spearmint plant have been traditionally employed to alleviate stomach aches and treat diarrhea (Amiri and Joharchi, 2013; Buso et al., 2020). Similarly, leaves of spearmint have long been used in the traditional ayurvedic system in India (Sharma et al., 2012).

Spearmint also harbors a wealth of bioactive compounds that contribute to its pharmacological properties. Studies have demonstrated that spearmint possesses notable antimicrobial properties, including antibacterial and antifungal activities (Scherer et al., 2013; Shahbazi, 2015; Alaklabi et al., 2016; Abdul Qadir et al., 2017; Powers et al., 2018). Additionally, its bioactive components have demonstrated anti-inflammatory and antioxidant capabilities, suggesting potential applications in the fields of immune system modulation and oxidative stress management (Dhifi et al., 2013; Mogosan et al., 2017; Bardaweel et al., 2018; Kehili et al., 2020).

On an industrial scale, the essential oil extracted from spearmint is used to flavor a range of products, including toothpaste, candles, candies, and jellies. Carvone, a principal component of its essential oil, imparts its characteristic flavor (Mahboubi, 2021). Beyond its medicinal and industrial roles, spearmint is renowned for its aromatic qualities and is widely embraced in culinary settings, enhancing the flavors of various foods and beverages (Mahendran et al., 2021).

2.4.2 Lemon Balm (*Mellissa officinalis* L.)

Melissa officinalis L, also known as lemon balm, is a bushy herbaceous perennial, belonging to the Lamiaceae family that can grow to a height ranging from 30 to 150 cm (Ashori et al., 2011; Shakeri et al., 2016) (Figure 2.4). Its square stems may exhibit a pubescent or hairy texture. The leaves, which are ovate and possess a medium green hue, are characterized by a wrinkled appearance. Measuring 2 to 8 cm in both length and width, the leaves feature crenate margins and a textured surface. Delicate, two-lipped, white flowers emerge in the leaf axils throughout the summer, showcasing colors ranging from pink to red, often with a white or yellow undertone (Shakeri et al., 2016).

Figure 2.4 Aerial parts of *Melissa officinalis* L. Figure adopted from Ashori et al., 2011.

Lemon balm a perennial herbaceous plant, originates from southern Europe, the Mediterranean Basin, Iran, and Central Asia. It has successfully naturalized in various regions worldwide, including North America (Miraj et al., 2017). Recognized for its distinct lemon fragrance, the plant produces petite white flowers with shades ranging from pink to red, white, or yellow. Lemon balm is renowned for its abundance of biologically active compounds, comprising terpenoids, phenolic acids, tannins, and essential oils. Leveraging these compounds, Lemon balm is valued for its therapeutic effects (Petrisor et al., 2022).

Lemon balm boasts a rich history in traditional medicine across various cultures. In traditional Moroccan medicine, it serves as a tranquilizing medicine, antispasmodic, and heart tonic (Merzouki et al., 2000; Bounihi et al., 2013). Widely documented traditional uses of lemon balm are prevalent in European countries, the Mediterranean region, and Middle Eastern countries. Its applications in traditional medicine encompass treating gastrointestinal tract disorders, anxiety, and functioning as a digestive, carminative, antispasmodic, sedative, analgesic, tonic, and diuretic agent (Miraj et al., 2017; Draginic et al., 2021). Contemporary pharmacological studies have substantiated many of the traditional uses of lemon balm, positioning it as a potential therapeutic resource for a broad spectrum of ailments (Shakeri et al., 2016). For example, methanolic extract of lemon balm displayed antianxiety effect by GABA-T inhibitory activity in an *in vitro* study on rat brain (Awad et al., 2009). Series of studies have also indicated that essential oils of lemon balm possess antioxidant properties (Bayat et al., 2012, p. 2015; Carocho et al., 2015; Luño et al., 2015).

Beyond medicinal uses, lemon balm finds its way into culinary practices, being utilized as a vegetable and for flavoring various dishes. The plant's therapeutic effects are attributed to its richness in biologically active compounds, including geranial, neral, citronellal, and geraniol, along with triterpenes like ursolic acid and oleanolic acid, and phenolic compounds such as rosmarinic acid and caffeic acid (Draginic et al., 2021). In summary, lemon balm emerges as a

versatile plant with significant potential, spanning both traditional medicine and diverse industrial applications.

2.4.3 Basket Plant (*Callisia fragrans* (Lindl.) Woodson)

Callisia fragrans, commonly known as the Basket Plant or False Bromeliad, is a diminutive perennial characterized by a rosette growth form from the Commelinaceae family. Its leaves, measuring 15 to 25 cm in length, exhibit a waxy green color with a subtle purplish tint on the undersides (Yarmolinsky et al., 2010; Graveson, 2022) (Figure 2.5). Tightly overlapped at the base, these leaves feature creamy strips and may take on a more pronounced purple hue when exposed to ample light. Trailing stems extend either horizontally over the ground or upward with support. The plant produces petite, fragrant white flowers arranged in long erect stems that rise above the foliage. These flowers, presented in clusters from the base of the plant, contribute to its distinctive and unusual appearance. Basket Plant is indigenous to Mexico and certain parts of South America (Beranová et al., 2022). Well-suited for cultivation in tropical or subtropical climates, this plant thrives in temperatures ranging from 3-5 °C. It can be cultivated as a potted or basket plant or utilized as ground cover.

Figure 2.5 Habitat, flowers and foliage of *Callisia fragrans* (Lindl.) Woodson. Figure adopted from Graveson 2022.

Traditionally, the Basket Plant has been harnessed for a multitude of medicinal applications. In Eastern Europe, the leaves of this plant find use in the treatment of skin diseases, with applications on burns to ease pain and facilitate the healing process (Yarmolinsky et al., 2010).

Renowned for its potential in addressing joint issues, the Basket Plant is believed to offer relief from both pain and inflammation. Additionally, it has served as a traditional remedy for cardiovascular diseases, bronchial asthma, and mucosal infections (El Sohafy et al., 2021). Beyond its traditional applications, the Basket Plant exhibits promising potential for industrial uses. Abundant in antioxidants, minerals, and vitamins, this plant emerges as a noteworthy raw material for the pharmaceutical industry (Chernenko et al., 2007). *C. fragrans* is not only esteemed for its medicinal attributes but also serves as an ornamental plant. Frequently cultivated as a houseplant, it is admired for its visual charm, featuring a distinctive appearance reminiscent of a bromeliad with a graceful, draping stem (Beranová et al., 2022). However, there is limited information regarding its utilization in the food industry.

2.4.4 Mini melon (*Melothria scabra* Naudin)

Melothria scabra, is a perennial creeping herbaceous plant. Referred to by various names such as Cucamelon, Chebbr, Mini melon, Mexican Sour Cucumber, Sour Gherkin, Mouse Melon, Pepquinos, or, Mini Cucumber, *M. scabra* is a member of the diverse Cucurbitaceae family (Daniel Hulse, 2022). The plant is native to Mexico and Central America but is distributed across Africa, Asia, Europe, and North America (Poot et al., 2008; Roberts et al., 2018; Kamaruddin et al., 2021). The plant exhibits distinctive characteristics, showcasing petite white or yellow flowers that give rise to a small edible fruit adorned with striped patterns reminiscent of a watermelon (Fernández-Cusimamani et al., 2023) (Figure 2.6). The fruits offer a multitude of applications, as they can be enjoyed in their natural form, or transformed into pickles or utilized as vegetables in various culinary preparations.

Traditionally, the plant's fruits and leaves are utilized for reducing blood pressure, while in Pakistan, the plant serves as a mosquito repellent (Fernández-Cusimamani et al., 2023). Studies indicate its antioxidant activity, suggesting a potential role in neutralizing harmful free radicals (Kamaruddin et al., 2021). Preliminary findings propose anti-diabetic and hypoglycemic properties, implying a potential application in managing diabetes (Govindula et al., 2019). The plant also shows promising anti-cancer properties, with high concentrations of Arginine and Citrulline, both known for their anti-cancer properties (Roberts et al., 2018; Hartman et al., 2019). Despite being listed as a minor crop species, *M. scabra* has gained popularity among agriculturalists and researchers due to its appealing appearance, distinctive flavor and its potential medicinal properties.

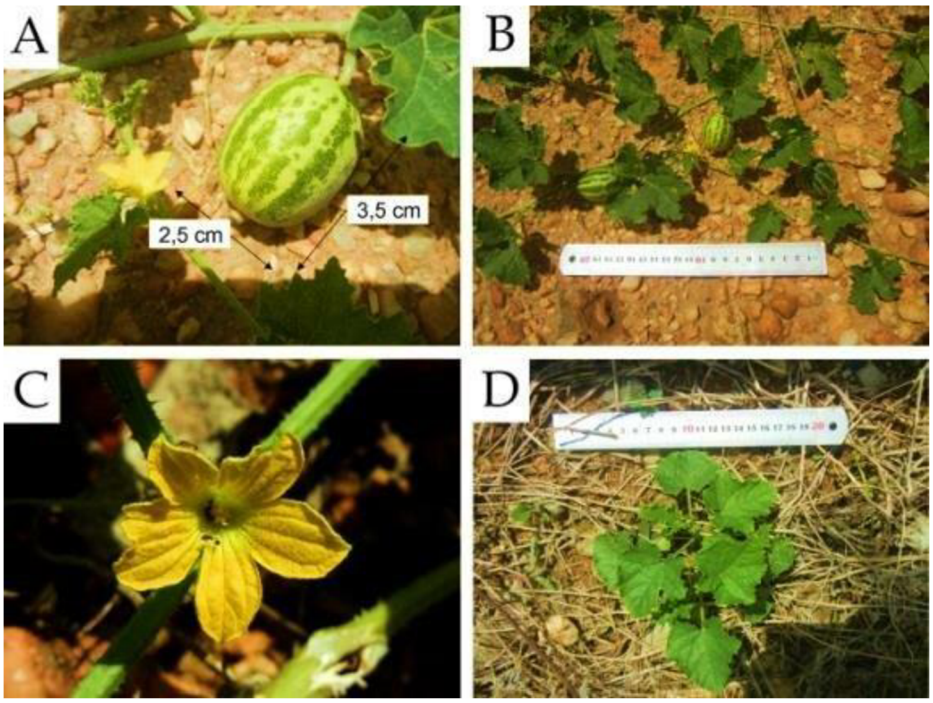

Figure 2.6 Figure exhibiting A) fruits, B) plant growth pattern, C) flower, and D) seedling of *Melothria scabra* Naudin. The figure was adopted from Kamaruddin et al., 2021.

2.4.5 Grapevines (*Vitis* spp.)

Grapevines, belonging to the *Vitis* genus, are perennial, woody climbing vines. The genus *Vitis* consists of 60-70 accepted species (Fortes and Pais, 2016; Keller, 2020). Some of the notable species among these includes *Vitis vinifera* that originated in southern Europe and southwestern Asia, *Vitis aestivalis* in the Eastern United States, *Vitis amurensis* in Asia, *Vitis arizonica* in Arizona, Utah, Nevada, California, New Mexico, Texas, and Northern Mexico, and *Vitis berlandieri* in southern North America (Kadir, 2006; Nassiri-Asl and Hosseinzadeh, 2009; Wan et al., 2013; Johnson and Hilsenbeck, 2016, p. 202120; Chen et al., 2018; Péros et al., 2021) (Figure 2.7). Grapevines are one of the major horticultural crops that are cultivated across the globe for its diverse range of uses (Zhang et al., 2021). They feature paper-like bark, alternate simple leaves of varying shapes and sizes, and tendrils used for climbing. Generally, grapevines have paniculate flowers that are deciduous at anthesis (Dhekney et al., 2020). Predominantly found in temperate regions of the Northern Hemisphere, Grapevines (*Vitis* spp.) are distributed in North America and eastern Asia (Fortes and Pais, 2016).

Grapevines play a crucial economic role as a source of grapes for consumption and winemaking. The fruit of grapevines is utilized for making jams, jellies, and other food products, contributing to dietary diversity (Bharati et al., 2023b). Grapevines harbor a plethora

of bioactive compounds, comprising linoleic acid, vitamin E, vitamin C, flavonoids, resveratrol, and procyanidins. The diverse array of these compounds imparts grapevines with a spectrum of pharmacological attributes, encompassing antioxidant, anti-inflammatory, anti-ulcer, anticancer, and antimutagenic activities (Ali et al., 2010).

Figure 2.7 Native geographical distributions of some of the major members of the *Vitis* genus. The figure was adopted from Wan et al., 2013.

3. Aims of the thesis

The current thesis aims to explore the potential of *in vitro* polyploidization as a biotechnological breeding approach in enhancing the economically important traits of plants with medicinal and therapeutic potential.

- To induce autopolyploid of selected medicinal plants, including spearmint, lemon balm, basket plant, and mini melon through *in vitro* polyploidization.
- To assess the potency of oryzalin as an antimitotic agent in the studied medicinal plants under *in vitro* conditions.
- Investigate the morphological, biochemical, anatomical, and physiological changes induced by synthetic polyploidization in the studied medicinal plants.
- To assess the genomic stability of the induced polyploids over time under *in vitro* and *ex vitro* conditions.
- Investigate the perspective of chromosome doubling in *Vitis* spp., addressing potential challenges, and integrate genomic selection and multi-omics as a cohesive strategy to overcome associated challenges in polyploidization of *Vitis* spp.

4. Hypotheses of the thesis

- The application of oryzalin will lead to the generation of polyploid genotypes of spearmint, lemon balm, basket plant, and mini melon through *in vitro* polyploidization.
- Polyploid genotypes of induced polyploids obtained through *in vitro* polyploidization will have enhanced morphological, biochemical, anatomical, and physiological changes compared to the control genotypes.
- Polyploid genotypes of induced polyploids obtained through *in vitro* polyploidization will have a stable ploidy level over time.
- The investigation will highlight the potential of chromosome doubling in *Vitis* spp., address the associated challenges, and explain how genomic selection integrated with multi-omics can be a cohesive strategy.
- Investigate the perspective of chromosome doubling in *Vitis* spp., addressing potential challenges, and integrate genomic selection and multi-omics as a cohesive strategy to overcome associated challenges in polyploidization of *Vitis* spp.

5. Oryzalin induces polyploids with superior morphology and increased levels of essential oil production in *Mentha spicata* L

Adapted from: **Bharati, R.,** Fernández-Cusimamani, E., Gupta, A., Novy, P., Moses, O., Severová, L., Svoboda, R., Šrédl, K., 2023. Oryzalin induces polyploids with superior morphology and increased levels of essential oil production in *Mentha spicata* L. Industrial Crops and Products 198, 116683. https://doi.org/10.1016/j.indcrop.2023.116683

This chapter explores the potential of oryzalin mediated in vitro polyploidization in inducing autopolyploid in Mentha spicata L. and assess its impact on the morpho physicochemical properties with special emphasis on essential oils.

Author contribution: Rohit Bharati was involved in conceptualization, investigation, methodology, writing original draft, writing review & editing, formal analysis.

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Abstract

Efficacy of oryzalin to generate novel genotypes of *Mentha spicata* L. with superior morphology and enhanced levels of essential oil via *in vitro* polyploidization was assessed. Micropropagated nodal segments were treated with oryzalin at 20, 40, and 60 μM for 24 and 48 h. The survival rate of the nodal segments dropped significantly with the increasing concentration and duration of oryzalin. Further, the ploidy level of the survived plants was confirmed using flow cytometry and chromosome counting. A total of six polyploids ($2n$ = 6x = 72) and six mixoploid plants were obtained. The highest frequency of polyploids (8%) was induced in treatment where 40 μM oryzalin was applied for 48 h. The obtained polyploids exhibited significantly larger, thick, dark green leaves with elongated stomata compared with the control genotype. The polyploid plants also displayed higher moisture content and vigorous lateral growth. The essential oil yield of hexaploid *M. spicata* exhibited a 48.85% increase, rising from 1.74% in control to 2.59% (*v/w*) in genotype P3. The major components of essential oils were carvone and limonene, which also increased significantly compared with the control genotype. The nutrient profile of the studied genotypes was influenced vastly and showed significant variations. Overall, the employed methodology using oryzalin as an antimitotic agent for polyploid induction in *M. spicata* was found to be effective. Despite relatively low polyploid induction frequency, the obtained polyploid genotypes demonstrated various superior agronomical traits. The genotypes obtained could serve to aid the commercial demands.

Keywords: Chromosome doubling; Essential oils; *In vitro*; Oryzalin; Polyploidization; Spearmint

5.1 Introduction

Mentha spicata L., spearmint, or just common mint, is a perennial herb belonging to the Lamiaceae family. From time immemorial, it has been widely used for its ethnopharmacological properties across the globe. For instance, in traditional Iranian medicine, spearmint leaves and aerial parts have been used to remedy flatulence and digestive disorders (Babaeian et al., 2015, Mahboubi, 2021), whereas it has been used to cure cholera, dysentery, diarrhea, and urine retention in India and Nepal (Adhikari et al., 2019, Upadhyay et al., 2010). Several recent studies have also reported spearmint to possess antibacterial, antifungal, antidiabetic, antioxidant, anticancer, anti-inflammatory, and antioxidant properties (Bardaweel et al., 2018, Ekhtelat et al., 2019, Farid et al., 2018, Kehili et al., 2020, Mahendran et al., 2021, Piras et al., 2021, Torres-Martínez et al., 2019). These properties are primarily attributed to essential oils present in spearmint that contain various aromatic compounds like carvone, limonene, carveol, dihydrocarvone, dihydrocarveol, and dihydrocarvyl acetate, which also contributes to the characteristic aroma of the plant (Mahendran et al., 2021). Besides their ethnopharmacological uses, these essential oils have various applications in the agricultural, medicinal, sanitary, food, and beverage industries (Mahendran et al., 2021, Zhang et al., 2022). Considering these advantages, the demand for these oils is on the rise and will continue to rise in the coming future. However, the average essential oil yield in *M. spicata* is between 0.04% and 2.1% (*v/w*) (Mahendran et al., 2021), which is relatively low considering the rising demand. Climate change, seasonal variation, and low-yielding varieties further complicate this scenario.

Numerous attempts have been made using traditional and biotechnological approaches in plant breeding for crop improvement. Synthetic polyploidization or chromosome doubling is one such approach, utilizing antimitotic agents to obtain genotypes with superior agronomical traits. It is commonly induced by compounds having the ability to hinder cell division. For that, colchicine is predominantly used owing to its thermostability and water-solubility (Eng and Ho, 2019, Touchell et al., 2020). Although several side effects of colchicine have been reported, like sterility, and gene mutation, and it often causes toxicity to humans (Dhooghe et al., 2011, Niazian and Nalousi, 2020). Additionally, it is relatively required in higher concentrations due to its low binding affinity to plant microtubules (Dhooghe et al., 2011, Touchell et al., 2020). As an alternative, mitosis-inhibiting herbicides like oryzalin are being utilized, which have fewer side effects compared with colchicine and possess a higher affinity toward plant tubulin (Beranová et al., 2022). Oryzalin has been successfully utilized in

obtaining polyploids of various economically important plants such as *Thymus vulgaris* (Homaidan Shmeit et al., 2020), *Cannabis sativa* (Parsons et al., 2019), *Allium hirtifolium* (Farhadi et al., 2022) and some blackberry species (Rubus spp.) (Sabooni et al., 2022). The antimitotic compounds could be applied to plants either under *ex vitro* or *in vitro* conditions. Although *ex vitro* conditions offer convenience, *in vitro* conditions provide better control and efficacy, hence are the preferred choice over *ex* vitro treatment for polyploid induction.

Polyploid plants produced by synthetic polyploidization often outperform their diploid counterparts. Generally, polyploids achieve larger leaves and stem size, increased levels of primary and secondary metabolites, and increased tolerance to biotic and abiotic stresses (Gantait and Mukherjee, 2021, Touchell et al., 2020). Some examples include increased levels of alkaloids in *Datura stramonium* (Berkov and Philipov, 2002), Phytophthora tolerance in the *Anemone sylvestris* (Šedivá et al., 2019), and salt tolerance in *Malus prunifolia* (Jin et al., 2022). Recently, several studies have also utilized synthetic polyploidization to increase the essential oils of various medicinal and aromatic plants (Homaidan Shmeit et al., 2020, Iannicelli et al., 2016, Moetamedipoor et al., 2022, Prasath et al., 2022). Interestingly, studies have also achieved novel metabolites in polyploids of these aromatic and medicinal herbs.

Considering these findings and potential prospects of synthetic polyploidization, it would not be erroneous to hypothesize that it could be an excellent tool to develop genotypes with superior morphology and higher essential oil contents in *M. spicata*. However, despite the medicinal and economic importance, it is surprising that no previous studies have explored the scope of synthetic polyploidization in *M. spicata* toward essential oil yield enhancement. Interestingly, oryzalin has never been tested for its efficiency in inducing polyploids in any of the *Mentha* spp. Hence, this study aims to determine the potential of oryzalin to generate polyploid genotypes of *M. spicata* through *in vitro* polyploidization. This study also aims to examine the influence of polyploidization on the essential oil yield and other morphological, anatomical, and biochemical attributes of *M. spicata*.

Previous studies have established polyploidization protocols in several medicinal and aromatic herbs, although the protocols are rather species-specific and may not be effective on other species. Thus, proper species-specific method development for polyploidization is a prerequisite to achieving polyploids effectively (Julião et al., 2020).

The results obtained from the current study will form a basis for future breeding attempts toward essential oil yield enhancement in medicinal and aromatic herbs through polyploidization. Additionally, it may also serve as an optimized protocol for *in vitro* polyploidization of *M. spicata* and other related species in order to achieve improved genotypes.

5.2 Materials and methods

5.2.1 Plant material acquisition, *in vitro* establishment, and multiplication

Mentha spicata (2 *n* = 3x = 36) plant samples were obtained from the maintained medicinal plant collection at the botanical garden of the Faculty of Tropical Agrisciences, Czech University of Life Sciences Prague, Czech Republic. *M. spicata* obtained was triploid with 12 basic number of chromosomes (x = 12) (Harley and Brighton, 1977). The samples were maintained at an average temperature of 23 °C and 70–80% relative humidity under greenhouse conditions. Stems containing no less than 5 nodes were selected and were washed under running tap water for 10 mins. Firstly, nodal segment explants were disinfected with 70% ethanol (*v/v*) for 2 mins, followed by immersing and continuously stirring them in a 1% solution of sodium hypochlorite (*v/v*) (NaClO, commercial bleach-SAVO) containing two drops of tween 20 for 10 min. Further, explants were rinsed thrice with sterile double distilled water and transferred to a 250 mL Erlenmeyer flask containing 50 mL (approx.) of MS (Murashige and Skoog, 1962) basal medium supplemented with 3% sucrose, 0.8% agar, and the pH was adjusted to 5.7 ± 0.1. The cultures were maintained in a growth room at 24/20 ± 1 °C (day/night) and relative humidity between 65% and 70%. Photoperiod was maintained at 16/8 h (light/dark) under white fluorescent lamps (3800 lux) (51.3 µmol m^{-2} s^{-1}). Developed plants were propagated by subculturing of nodal segments every 14 days until sufficient plants were obtained for polyploid induction.

5.2.2 Polyploid induction

Nodal segments of *in vitro* grown plants (10–15 mm approx.) were grown on the MS medium for 48 h in beakers prior to the treatments. Then, three concentrations of oryzalin (20, 40, and 60 µM) were prepared by dissolving in a 1% Dimethyl sulfoxide (DMSO) solution. Subsequently, under a sterile laminar flow box, prepared oryzalin solutions were poured onto the inoculated explants until all explants were submerged. The experiment involved six treatments (T1-T6) using oryzalin at varying concentrations and exposure durations. The treatments were as follows: 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). A control variant (Control), where explants were subjected to basic MS medium without any oryzalin treatment was also used. A total of 50 nodal segment explants were utilized per treatment. Subsequently, all the treated explants were removed from oryzalin solutions and rinsed three times with sterile double distilled water. The washed explants were then reinoculated on MS medium and maintained for six months with regular subculturing every 30 days. After every three months, the ploidy level was assessed using a flow cytometer (Partec GmbH, Münster, DE).

5.2.3 *Ex vitro* transfer

After maintaining for six months in *in vitro* conditions, control and polyploid plants were transferred to the greenhouse with an average temperature of 24 °C and relative humidity between 60% and 70%. Plants were planted in small pots (5 × 5 cm) containing soil and perlite in a 3:1 ratio, then kept under transparent polythene covering (primary hardening) for seven days. The plants were then transferred to larger pots and kept in the greenhouse without polythene covering for another 15 days. Finally, the plants were transferred to the field conditions for 60 days before data and samples were collected for further analysis.

5.2.4 Morphological analysis

Morphological growth parameters of control and hexaploid genotypes were evaluated to assess the effects of polyploidization. The growth parameters included: number of shoots, shoot length, number of nodes per shoot, internodal distance, number of leaves per shoot, leaf area, leaf thickness, and stem thickness.

5.2.5 Chlorophyll estimation

Chlorophyll estimation was carried out by a previously reported protocol (Mosa et al., 2018) with slight modifications, where fresh leaf samples from triploid control and hexaploid plants were collected and crushed using liquid nitrogen in mortal and pestle until a fine powder was achieved. From the grounded powder, 300 mg was suspended in 5 mL of 80% acetone and kept in a shaker for 15 min under dark conditions. Thereafter, the resulting mixture was centrifuged for 15 min at 4 °C at 3000 rpm. The supernatant was collected and diluted using 80% acetone in a 1:5 ratio. Finally, absorbance was measured for chlorophyll a (663 nm) and chlorophyll b (645 nm) at two wavelengths using a spectrophotometer, and the values generated were used to

calculate the total chlorophyll content of triploid control and hexaploid plants according to a previous study of Manzoor et al. (2018).

5.2.6 Stomatal observation

The size and density of stomata from triploid control and hexaploid plants were compared using the nail polish impression method (Grant and Vatnick, 2004, Parsons et al., 2019) with slight modifications. Transparent nail polish was applied to the abaxial side of the leaves and left to dry. Thereafter, impressions were taken using transparent tape. The size and density of stomata were recorded under a light microscope at 60× magnification. A total of 20 fields were investigated for each variant.

5.2.7 Flow cytometry analysis

A small leaf segment (1 cm^2 approx.) was taken in a Petri dish and chopped using a sharp razor blade in 500 µL of Otto I buffer (0.1 M $C_6H_8O_7$, 0.5% Tween 20). The chopped crude suspension was then filtered through a 50 µm nylon mesh. Further, 1 mL solution of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_2O$) containing 2 µg/mL of fluorescent dye (DAPI) was added to the filtered crude suspension. A minimum of 10000 nuclei per sample were measured using a Partec PAS flow cytometer (Partec GmbH, Münster, DE). Histograms of relative DNA content were evaluated and recorded using the Flomax software package (Version 2.3).

5.2.8 Chromosomes counting

Chromosome counting was carried out by a previously reported protocol (Beranová et al., 2022) with slight modifications in both control and polyploid genotypes. Fresh root tips (1 cm approx.) were collected between 7 and 8 o′clock in the morning and kept in a saturated solution of Paradichlorobenzene for 2 h at room temperature. Thereafter, root tips were rinsed with distilled water thrice and put into a freshly prepared solution of ethyl alcohol and acetic acid (3:1) for 60 min at room temperature. The roots were removed from the solution and washed three times again with distilled water prior to the hydrolysis and staining steps. The root tips were then incubated in a 1 N HCl solution at 60 °C for 10 min, followed by staining using Schiff reagent for 1 h. Finally, root tips were excised further (0.2 cm approx.) and placed on a glass slide with a drop of 2% orcein-acetic solution and visualized under a BX51 Olympus light microscope (Olympus Optical Co., Tokyo, Japan) at 100× magnification. For each genotype, a

minimum of 5 root tips were randomly selected and chromosomes were counted across 10 microscopic fields per root tip to ensure accurate representation.

5.2.9 Essential oils extraction

Fresh aerial parts were harvested from triploid control and hexaploid plants after 60 days of cultivation and dried in a drying oven (UF55plus, Memmert, USA) at 30 °C until a constant dry weight was achieved. Dried samples were then run through hydro-distillation using a Clevenger-type apparatus for three hours. Thereafter, the extracted oils were stored at 4 °C in airtight glass vials until further utilized for Gas chromatography-mass spectrometry (GC/MS) analysis.

5.2.10 GC/MS analysis of essential oils

Essential oil components were identified using an Agilent 7890A gas chromatograph (GC) coupled with an Agilent 5975C single-quadrupole mass detector and quantified using an Agilent 6890A GC with flame ionization detector (FID). Both chromatographs were equipped with a non-polar column HP-5MS (30 m × 250 µm × 0.25 µm) (Agilent, Santa Clara, CA, USA). Samples were diluted 1:1000 in hexane (VWR, Stribrna Skalice, Czech Republic), and one µL was injected in a 12:1 split ratio into the inlet heated to 250 °C. The initial oven temperature was set to 60 °C for 3 min, then it was gradually increased at the rate of 5 °C/min up to 231 °C and kept constant for 10 min. Helium was used as a carrier gas at the flow rate of 1 mL/min. The FID detector was heated to 250 °C. The MS analysis was carried out in full scan mode, whereas the electron ionization energy was set to 70 eV. The relative percentage content was expressed as the ratio of individual peak area to the total area of all peaks. The identification of the essential oil components was based on a comparison of mass spectra and Kovat's retention indices with the National Institute of Standards and Technology Library (NIST, USA) and literature (Adams, 2007). The identification of 13 components was further confirmed with authentic standards, all obtained from Sigma–Aldrich (Prague, Czech Republic).

5.2.11 ICP-MS analysis of micro and macronutrients

Approximately 200 mg of dried samples were weighed and placed in a quartz vessel, and 4 mL of HNO_3 (Analpure®, Analytika, Czech Republic) and 2.0 mL of H_2O_2 (Rotipuran®, Carl Roth, Germany) were added. The prepared samples were then digested in a closed vessel microwave system for 20 mins at 180 °C. The digested solutions were transferred to

50 mL polypropylene tubes and filled with Milli-Q water (≥ 18.2 MΩ cm-1; MilliQ system, Millipore, SAS, France) up to a final volume of 45 mL. Then the elemental concentration of some macro and micro elements were analyzed by an inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7700×, Agilent Technologies Inc., USA). A certified reference material was included for quality assurance, namely Peach leaves (SRM-1547, NIST). Three biological and three technical replicates were taken for each genotype.

5.2.12 Statistical analysis

The data obtained from the morphological, biochemical, and anatomical parameters of the control and all the induced polyploid genotypes were analyzed using one-way ANOVA (analysis of variance) with Tukey's honest significant difference post hoc comparison test. All tests were carried out at $p < 0.05$ in Microsoft excel 2021 software package. The graphs presented were plotted using GraphPad Prism (Version 9.4.0).

5.3 Results

5.3.1 Survival rate, polyploidy induction, and ploidy stability

MS growth medium without any plant growth regulators was sufficient for the micropropagation of *M. spicata* from nodal segments. Upon propagation in an adequate amount, a total of 300 nodal segments were subjected to oryzalin treatments for polyploid induction. Of these, 35% of the plants survived the treatment. The highest and lowest survival rate was observed in treatment T1 (48%) and T6 (22%), respectively (Figure 5.1). It was observed that the survival rate decreased significantly with the increasing concentration and duration of oryzalin (Figure 5.1). The histogram obtained from the flow cytometric analysis differentiated polyploid genotypes from the control ones (Figure 5.2A-D). By assessing the ploidy level, 7 polyploid and 6 mixoploid plant genotypes were identified among the surviving plants. Samples with multiple peaks were considered mixoploids. The highest polyploid induction rate was found in treatment T4 (8%) and the lowest in T1, T5, and T6, all with just a 2% polyploid induction rate (Figure 5.1). On the other hand, treatments T2 and T3 were found to be ineffective, with a 0% polyploid induction rate. Out of 7 polyploids, one did not generate a viable root system and died, while six mixoploids obtained reverted to their natural ploidy levels (triploid) during subculturing. The ploidy levels of the treated plants were also assessed by chromosome counting. It was confirmed that chromosomes in treated plants were doubled ($2n = 6× = 72$) when compared with the triploid control plants ($2n = 3× = 36$)

(Figure 5.2A-D). Finally, the six obtained polyploids were labeled sequentially as P1-P6 and further evaluated for their morphological and biochemical characteristics.

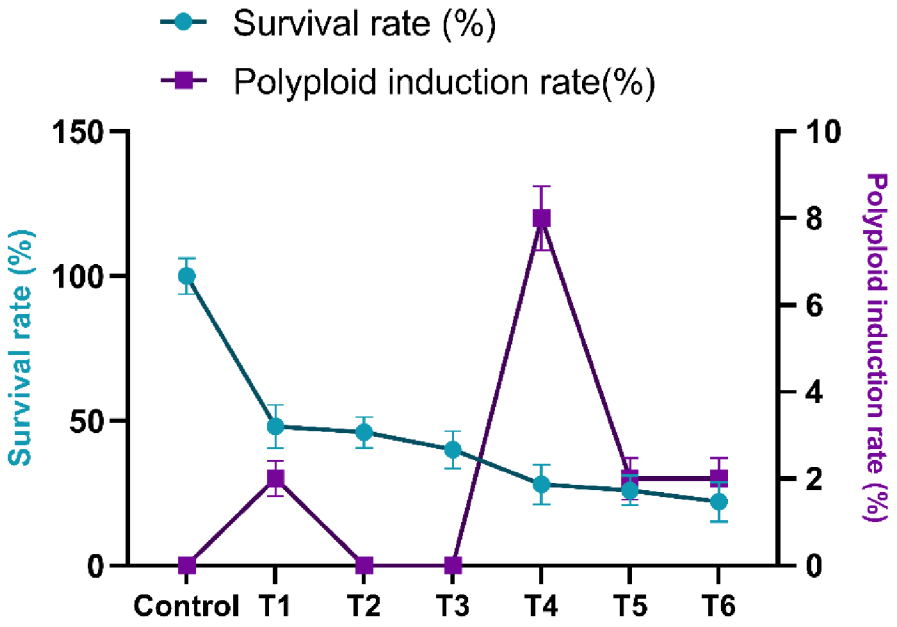

Figure 5.1 Effect of different concentrations and duration of oryzalin treatment on survival rate and polyploid induction rate in *M. spicata*.

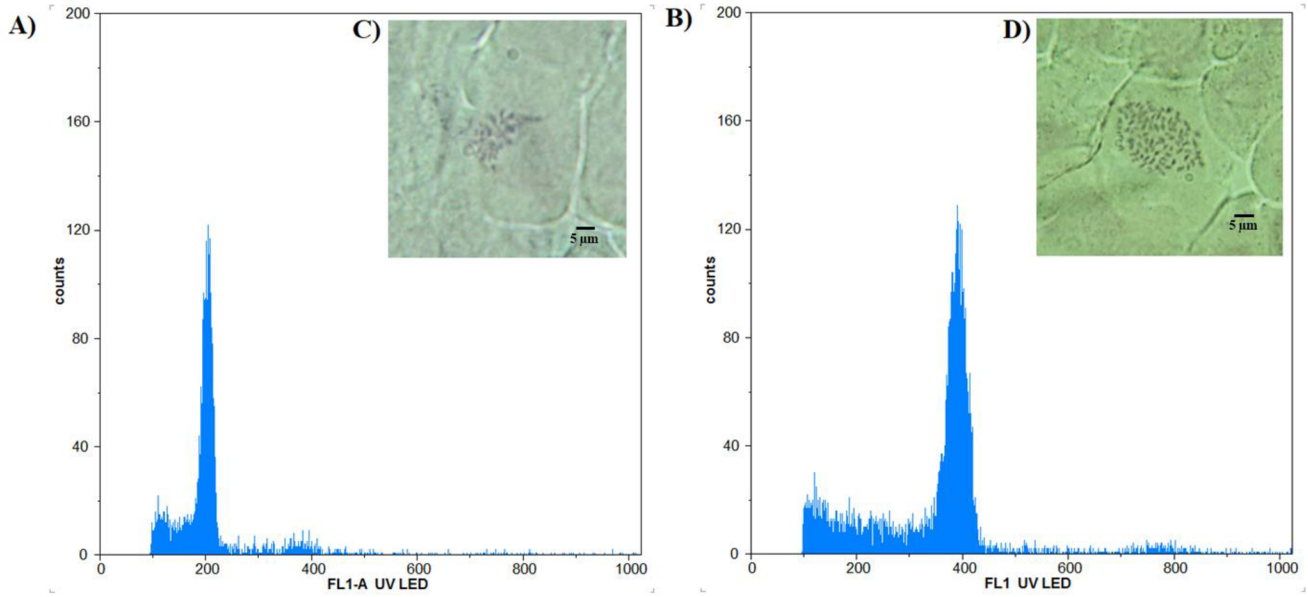

Figure 5.2 Flow cytometry analysis of *M. spicata* (A) triploid and (B) hexaploid plant, depicting relative DNA content along with chromosomes under 100x magnification for (C) triploid and (D) hexaploid plant.

5.3.2 Morphological and physiological characteristics

Significant differences in morphological characteristics were observed between the control and the induced polyploids (Figure 5.3), and the data are summarized in Table 5.1. The average number of shoots per plant increased significantly among all the polyploid plants except genotype P1. The highest increase was observed in genotype P6 (72.81%). Although the average length of the shoots among polyploids did not increase but was the same or significantly shorter when compared with the control plant (Table 5.1). No changes in the average number of nodes per shoot were observed among polyploid plants, compared with the control plant, except for P1 and P3, which were significantly lower and higher, respectively. Additionally, a decrease in the internodal distance was observed in P2 and P3, whereas the number of leaves per shoot did not exhibit any significant change compared with the control plant, except for P2, where it increased approximately 1.5 times (Table 5.1). Interestingly, the average leaf area among all the polyploids (except P1) increased and almost doubled in some genotypes (P2, P6) (Table 5.1) (Figure 5.4). Similarly, a significant increase in the leaf and stem thickness was also observed among polyploid genotypes compared with the control plants (Table 5.1). Although fresh biomass of the aerial parts decreased in some polyploid genotypes (P1, P3, P4), it increased significantly in P5 and P6. The dry weight in all the polyploid genotypes remained the same or significantly lower (P1-P3) than in the control, indicating some polyploids possess a higher amount of water content compared with the control genotype (Table 5.1).

Table 5.1 Growth characteristics of triploid control and induced polyploid genotypes (P1-P6) of *M. spicata*.

Variant	Number of shoots	Shoot length (cm)	Number of nodes per shoot	Internodal distance (cm)	Number of leaves per shoot	Leaf area (cm^2)	Leaf thickness (mm)	Stem thickness (mm)	Wet weight (g)	Dry weight (g)
Control	33.07±3.70d	23.40±2.94a	8.00±1.60b	2.23±0.29a	18±3.20bc	8.17±1.32c	0.52±0.11c	2.36±0.27c	104.25±7.25bc	22.43±1.12a
P1	34.15±4.23d	15.40±1.17c	6.66±1.28c	1.93±0.35ab	15.33±2.57c	8.12±1.15c	0.73±0.22b	2.61±0.58bc	81.03±9.02de	10.59±0.97c
P2	56.15±3.34a	22.25±2.33a	12.55±1.63a	1.37±0.18c	27.1±3.25a	13.02±1.66ab	0.8±0.15ab	2.83±0.37b	99.66±8.87c	14.5±1.17b
P3	47.38±2.72b	16.87±2.76c	8.10±1.87b	1.69±0.23b	18.2±3.74bc	12.52±2.10ab	0.93±0.19a	2.61±0.30bc	77.85±12.59e	12.27±0.83bc
P4	51.30±3.52bc	20.45±3.51b	9.30±2.00b	1.96±0.17ab	20.6±4.00b	10.89±1.98bc	0.89±0.18ab	2.94±0.46ab	88.22±6.61d	21.31±1.46a
P5	54.23±3.67ab	21.75±2.41ab	8.70±1.71b	2.23±0.24a	19.4±3.41b	11.34±2.19bc	0.86±0.20ab	3.36±0.61a	110.65±11.71ab	19.58±0.75a
P6	57.15±3.76a	21.27±2.47ab	8.95±1.72b	2.02±0.26a	19.9±3.43b	15.87±4.73a	0.88±0.22ab	2.92±0.45b	116.69±9.64a	19.96±1.75a

Different letters within the same column differ significantly (Tukey HSD Test, $p < 0.05$). The data presented are mean ± standard deviation (n=20).

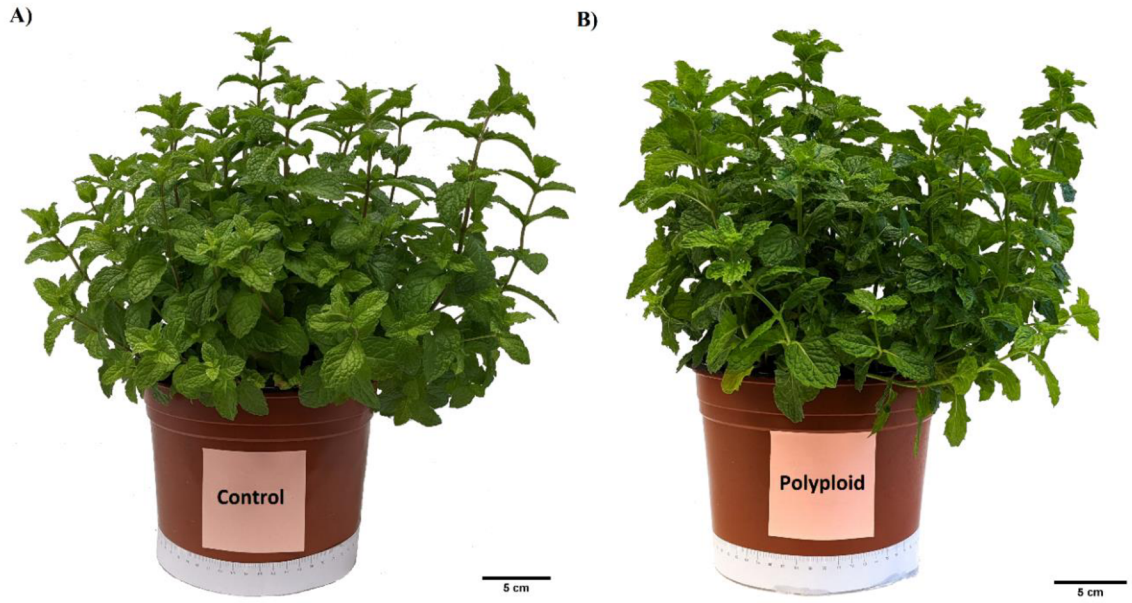

Figure 5.3 Phenotype of *M. spicata* (A) control triploid and (B) induced hexaploid plant.

Figure 5.4 Leaf shape and size of *M. spicata* (A) control triploid and (B) induced hexaploid plant.

5.3.3 Stomatal size and frequency comparison

Notable differences were observed between the stomatal characteristics of the control and induced polyploid genotypes (Figure 5.5A,B). The major change was observed in the length of the stomata among the polyploids (Figure 5.5B), where it increased significantly by 35.90% while the width remained unchanged when compared with the control genotype (Figure 5.5B, Table 4.2). On the other hand, the stomatal frequency decreased significantly among polyploids to nearly half per unit area (Table 5.2, Figure 5.5A,B).

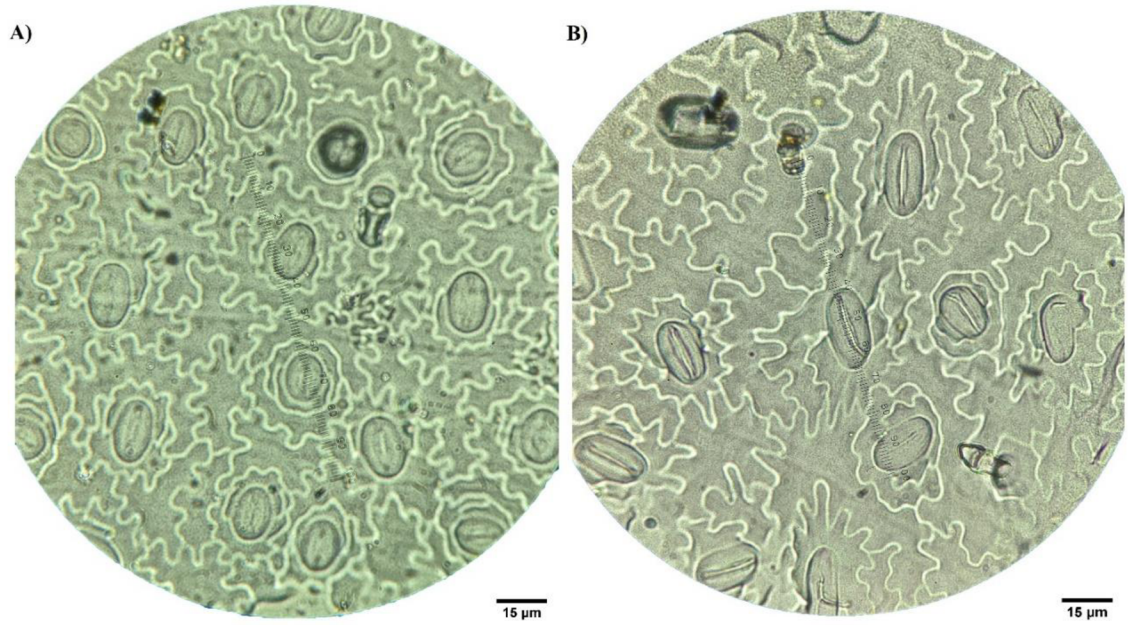

Figure 5.5 Stomata size and frequency in *M. spicata* (A) control and (B) induced polyploid plant.

Table 5.2 Comparison of stomatal characteristics between triploid control and polyploid genotypes of *M. spicata*.

Variant	Average number of stomata per magnification field (60x)	Average stomata length (µm)	Average stomata width (µm)
Control	18.38±2.34a	17.13±1.27b	12.27±0.55a
Polyploids (P1-P6)	10.9±1.79b	23.28±2.88a	12.03±0.70a

Different superscript letters within the same column differ significantly (Tukey HSD Test, $p < 0.05$) The data presented are mean ± standard deviation (n=20).

5.3.4 Chlorophyll content

The polyploid plants exhibited thicker leaves with a darker color (except for P1), indicating higher levels of chlorophyll contents. This was validated by estimating the total chlorophyll content. It was elucidated that all the polyploid genotypes attained higher levels of total chlorophyll content, except for P1 and P6, where it decreased and remained unchanged, respectively when compared with the control (Figure 5.6A). The highest chlorophyll content was recorded in P4 (1.57 mg/g FW), which was a 70.65% increase compared with the triploid genotype (Figure 5.6A).

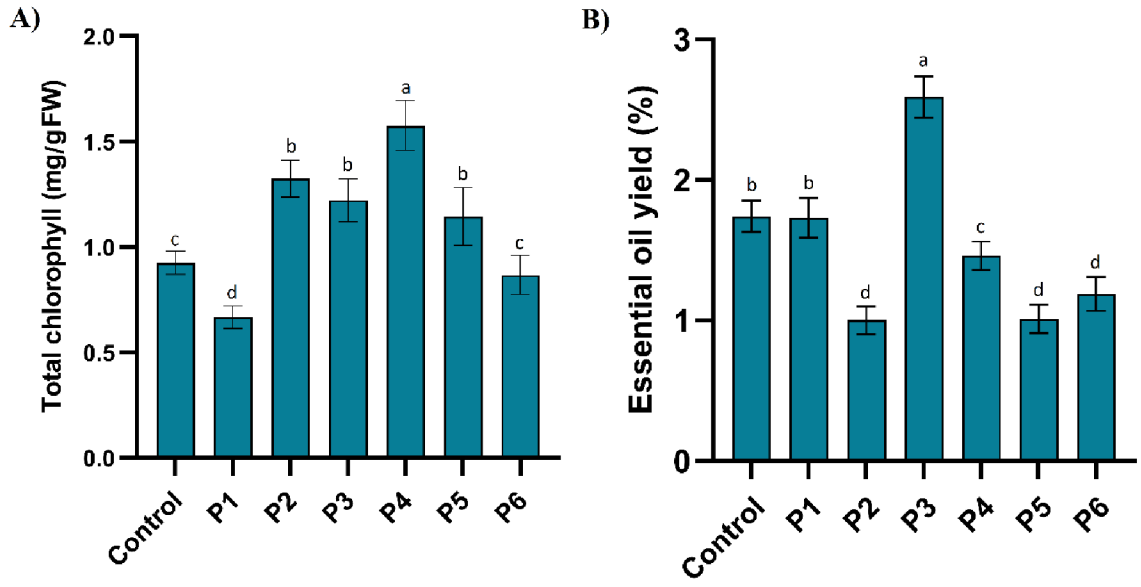

Figure 5.6 Total chlorophyll content (A) and essential oil yield (B) in control and induced polyploid (P1-P6) plants of *M. spicata*. Different letters on the vertical bars differ significantly (Tukey HSD Test, $p < 0.05$) (n = 20).

5.3.5 Essential oil yield and composition

The essential oil yields ranged from 1% to 2.59% (*v/w*) in polyploid plants, while the triploid control plant yielded 1.74% of essential oil. The highest essential oil yield was obtained in genotype P3 (2.59%) and the lowest in P2 (1%) (Figure 5.6B). In total, 27 components were identified in all samples representing 97.95–98.88% of the total composition of the oils (Table 5.3). The major compounds were carvone and limonene, which together constituted 85.13–

90.14% of the total composition among all the genotypes. Among the polyploids, carvone and limonene ranged between 55.24% (P4)– 73.83% (P1) and 15.99% (P6)– 32.70% (P4), respectively, whereas it was 68.20% for carvone and 19.06% for limonene in the control genotype (Table 5.3). The rest components didn´t exceed 1%, with the exception of myrcene, eucalyptol, germacrene D, and β-caryophyllene.

Table 5.3 Essential oil constituents of triploid (control) and polyploid genotypes (P1-P6) of *M. spicata.*

RI[a]	RI(lit)[b]	Compound	Content (%)[c]						
			Control	P1	P2	P3	P4	P5	P6
937	939	α-Pinene [S]	0.62	0.63	0.64	0.55	0.61	0.55	0.58
952	953	Camphene [S]	0.07	tr	0.10	0.05	0.09	0.08	0.06
976	976	Sabinene	0.44	0.39	0.45	0.40	0.49	0.42	0.39
979	980	β-Pinene [S]	0.81	0.07	0.85	0.71	0.87	0.72	0.68
992	991	Myrcene [S]	1.36	0.92	1.45	1.74	1.55	1.61	1.48
1005	1005	α-Phellandrene [S]	0.05	tr	tr	0.06	0.05	0.05	tr
1031	**1031**	**Limonene [S]**	**19.06**	**16.30**	**18.22**	**22.84**	**32.70**	**19.13**	**15.99**
1033	1033	Eucalyptol [S]	1.50	0.59	1.38	0.49	2.00	1.46	1.00
1041	1040	*Z*-β-Ocimene	0.52	0.52	0.59	0.59	0.69	0.58	0.34
1051	1050	*E*-β-Ocimene	0.16	0.15	0.17	0.19	0.22	0.14	0.09
1099	1098	Linalool [S]	0.22	0.34	0.21	0.30	0.23	0.25	0.25
1167	1165	Borneol [S]	0.25	0.03	0.34	0.08	0.36	0.30	0.26
1194	1192	Dihydro carveol	0.16	0.22	0.21	0.38	0.13	0.17	0.25
1196	1193	*Z*-Dihydrocarvone [S]	0.21	0.48	0.23	0.36	0.24	0.27	0.43
1219	1217	*E*-Carveol	0.15	0.46	0.33	0.12	0.08	0.13	0.28
1232	1229	*Z*-Carveol	0.37	0.23	0.49	0.39	0.29	0.21	0.37
1247	**1242**	**Carvone [S]**	**68.20**	**73.83**	**67.47**	**66.33**	**55.24**	**66.00**	**71.33**
1330	1325	iso-Dihydrocarvyl acetate	tr	0.05	tr	0.20	0.05	tr	0.09
1341	1339	Piperitenone	0.32	0.41	0.33	1.23	0.78	0.25	0.37
1364	1362	*Z*-Carvyl acetate	0.11	0.12	0.12	0.38	0.14	0.10	0.30
1366	1363	Piperitenone oxide	0.09	0.13	0.12	0.63	0.50	0.12	0.12
1385	1384	β-Bourbonene	0.57	0.49	0.72	0.14	0.24	0.54	0.57
1392	1391	β-Elemene	0.15	0.12	0.16	tr	0.06	0.21	0.18
1419	1418	β-Caryophyllene [S]	0.70	0.14	0.86	0.20	0.31	1.10	0.70
1454	1454	α-Humulene [S]	0.14	tr	0.17	0.05	0.07	0.19	0.12
1463	1461	*Z*-Muurola-4(15),5-diene	0.34	0.33	0.44	0.11	0.19	0.64	0.33
1481	1480	Germacrene D	1.67	1.13	1.82	0.33	0.69	2.69	1.96

Total identified	98.29	98.15	97.95	98.88	98.86	97.98	98.58

a: Kovat´s retention indices measured on HP-5MS column; b: retention indices from literature; c: relative percentage content based on total area of all peaks; S: Identification (based on comparison of mass spectra with NIST library) confirmed by co-injection with authentic standard; tr: trace amount (<0,05%).

5.3.6 Macro- and micronutrients content

Macro- and micro-nutrient contents varied among the studied genotypes. When compared with the control genotype, both the analyzed macronutrients, Calcium (Ca) and Magnesium (Mg), were found to remain unchanged or decreased significantly among the polyploids except for P1, where Mg increased (5.89%), as well as P6, where both (Ca and Mg) increased sharply (18.30% and 24.10% respectively) (Table 5.4).

Table 5.4 Macronutrient and micronutrient compounds analyzed from leaves of control and induced polyploids (P1-P6) of *M. spicata* plants (mg/kg).

Variants	Ca	Mg	Mn	Fe	Zn	B	Cu	Mo
Control	14,994.07±28 8.29bc	4,789.65±4 5.91c	719.79±1 0.63a	389.26±2 3.65a	144.91±3. 61b	33.91±1. 41c	11.11±0 .23c	0.54±0. 01c
P1	15,164±127.3 8b	5,072.03±9 1.50b	374.29±1. 72b	208.85±6. 78b	122.27±1. 54b	45.51±0. 16a	17.21±0 .07a	0.66±0. 01b
P2	10,804.14±38 .84d	4,150.95±2 5.35e	145.32±3. 43e	113.11±4. 60c	57.17±1.3 9c	27.69±0. 75d	7.43±0. 24e	0.05±0. 01f
P3	14,558.75±19 5.10c	4,557.55±1 5.05d	418.58±1. 86c	236.34±4. 90b	119.87±0. 75b	37.28±0. 49b	17.51±0 .06a	0.84±0. 02a
P4	10,155.07±15 5.98e	4,363.88±1 5.48d	152.75±2. 40e	143.32±2 8.91c	61.65±1.3 1c	24.70±0. 58e	6.25±0. 12f	0.21±0. 02e
P5	10,663.42±65 .4de	4,641.32±1 4.61cd	237.34±1. 54d	114.33±2. 08c	77.69±1.6 9c	26.81±0. 82de	9.60±0. 19d	0.17±0. 00e
P6	17,739.41±31 3.28a	5,944.22±1 51.22a	628.14±9. 81b	234.88±1 1.33b	176.14±2 9.22a	38.75±0. 62b	16.06±0 .28b	0.45±0. 03d

Different letters within the same column differ significantly (Tukey HSD Test, $p < 0.05$). The data presented are mean ± standard deviation (n=20).

Among the micronutrients, manganese (Mn) and iron (Fe) were highest in the control genotype when compared with the induced polyploids. Zinc (Zn) was the highest in the P6 genotype, while its content was lower or the same among other genotypes. Compared to the control, higher

content of boron (B) was recorded in polyploids P1 and P6, Copper (Cu) in P1, P3, and P6, as well as molybdenum (Mo) in P1 and P3.

5.4 Discussion

Synthetic polyploidization is an excellent tool to generate a wider pool of germplasm for plant breeding (Sattler et al., 2016). Polyploidization often leads to genotypes with larger organs, higher secondary metabolite production, and increased tolerance to biotic and abiotic stresses (Gantait and Mukherjee, 2021, Touchell et al., 2020). Polyploidization for crop improvement in the mint family has recently drawn the attention of researchers for its potency to achieve a higher level of secondary metabolite, especially essential oils. However, these attempts are still at a juvenile phase, considering the wide species diversity of the mint family, so extensive rational attempts would be required.

The use of antimitotic agents such as colchicine has been previously reported for polyploidization in *Mentha* spp., while oryzalin has never been tested for its efficacy in any of the *Mentha* spp. At the same time, synthetic polyploidization has never been carried out in *M. spicata*. Hence, polyploidization in *M. spicata* using oryzalin as an antimitotic agent was carried out for the first time. The present study elucidated that oryzalin at 40 µM for 48 h was the most effective treatment for chromosome doubling in this species since lower and higher concentrations used were either ineffective or had lower efficacy. The effectiveness of oryzalin as an antimitotic agent is well-established across numerous plant species. Moreover, it has become a preferred choice over commonly used colchicine due to its lower toxicity and higher affinity for plant tubulins (Beranová et al., 2022, Dhooghe et al., 2011). For the same reasons, oryzalin generally is required in comparatively small quantities for relatively similar or even better results with lesser toxicity. For instance, a study by Sakhanokho et al. (2009), tested the efficacy of colchicine and oryzalin for inducing polyploids in Ornamental Ginger (*Hedychium muluense*), and oryzalin at 60 µM was found to be more effective with 15% induction frequency compared with colchicine at 2.5 mM with 13%. Similar results have been reported in *Watsonia lepida* (Ascough et al., 2008), *Passiflora edulis Sims.* (Rêgo et al., 2011) and some of the *Vaccinium* species (Tsuda et al., 2013), where oryzalin has also been more effective than colchicine.

In the current study, in addition to *M. spicata* polyploids, an almost equal number of mixoploids (six) were obtained. It is worth noting that all mixoploids reverted to the natural triploid state

during subcultivations. This indicates that this species is quite unstable at the mixoploid state, so it prefers to remain in the triploid state. The instability at the mixoploid level has been reported previously in mojito mint, where the mixoploid plants also reverted to their natural ploidy state (Moetamedipoor et al., 2022). The obtained polyploids of *M. spicata* exhibited vigorous growth with larger organs. Polyploids exhibited increased shoot numbers and reduced internodal distance resulting in a bushier growth compared to the control genotype. Enhanced growth and morphology are well-reported consequences of polyploidization, also known as "gigas effect". For instance, in *Thymus vulgaris* (a member of Lamiaceae family), polyploidization caused a significant increase in leaf length, width, and thickness (Homaidan Shmeit et al., 2020). Similarly, leaf area after polyploidization in *Agastache foeniculum* L. increased significantly from 12.66 cm^2 to 22.16 cm^2 (Talebi et al., 2017). Significant changes were also obtained for the leaf area, leaf and stem thickness, where it increased and nearly doubled in the *M. spicata* polyploids (except P1). Although, these results are inconsistent with previous studies from other *Mentha* species, where leaf area and thickness did not change or even significantly decrease in the obtained polyploids (Moetamedipoor et al., 2022, Yu et al., 2013). The obtained improvements are significant for commercial cultivation, as polyploids of *M. spicata* can generate greater biomass per unit of land area, leading to a substantial boost in overall yield.

The size and frequency of stomata have frequently been used to confirm the polyploidization event, as these attributes are less influenced by environmental factors (Lourkisti et al., 2021, Moetamedipoor et al., 2022). Generally, polyploidization is accompanied by an increase in stomatal length and width (Eng and Ho, 2019, Omidbaigi et al., 2010), although it has also been frequently reported that stomatal frequency decreases with polyploidization (Ghani et al., 2014, Moetamedipoor et al., 2022, Talebi et al., 2017). In *M. spicata*, stomatal length increased significantly among the polyploid genotypes while the frequency decreased sharply.

Previously, it has been reported that the average essential oil yield in *M. spicata* ranges between 0.04% and 2.1% (*v/w*) (Mahendran et al., 2021). Mostly the essential oil yields among the polyploids in the current study decreased or did not change; however, the P3 genotype exhibited a record-high amount of essential oil (2.59%), which is a 48.85% increase compared with the control genotype. Similar results where essential yield increased significantly in induced polyploids have been reported in *Mentha* × *villosa* (Moetamedipoor et al., 2022) and *Thymus vulgaris* (Homaidan Shmeit et al., 2020). Due to this potential of increasing secondary

metabolites, synthetic polyploidization is gaining popularity in the plant breeding of medicinal herbs.

GC-MS analysis indicated that the essential oil of all the genotypes had two major components, carvone, and limonene. When compared with the control, carvone increased in P1 and P6, and limonene increased in P3 and P4 genotypes. Carvone and limonene are widely used natural products in the cosmetics, agriculture, food preservation, and pharmaceutical industries (Vieira et al., 2018, Pina et al., 2022). These compounds have been elucidated to possess antioxidant, antinociceptive and anti-inflammatory properties (Souza et al., 2013, Vieira et al., 2018, Zhao and Du, 2020, Pina et al., 2022). The increase of these compounds within the essential oils of the polyploids presents an exciting opportunity for the application of these genotypes across various industries. These results suggest the possibility that the novel genotypes of *M. spicata* resulting from polyploidization may have increased therapeutic potency, highlighting the need for further study in this area.

The induced genotypes displayed a wide variation in the macro and micronutrient profiles. Compared with the control genotype, the P6 genotype showed an increase in Ca and Mg by 18.30% and 24.10%, respectively. Similarly, a study on *Moringa oleifera* also reported a 20% increase in calcium content among the induced polyploid (Ridwan and Witjaksono, 2020). A recent study on polyploidization in *Callisia fragrans* analyzed the macro and micronutrients among the obtained polyploids, where potassium content showed almost a 100% increase among the polyploid and mixoploid genotypes obtained. At the same time, some genotypes showed higher amounts of calcium, while some exhibited significantly decreased levels (Beranová et al., 2022). The findings from current and previous studies indicate that polyploidization could be an effective strategy for obtaining genotypes with different nutrient profiles.

5.5 Conclusions

In the current study, chromosome doubling of *M. spicata* ($2n = 6\times = 72$) using oryzalin applied to the nodal segments generated novel polyploid genotypes ($2n = 6\times = 72$) with superior traits. Findings also indicate that spearmint at hexaploid ploidy level is stable and grows normally over time. The induced genotypes exhibited significantly large, thick, dark green leaves with elongated stomata and high chlorophyll contents. The polyploid genotype P3 was established as a superior genotype with a 48.88% increase in the essential oil yield, while genotypes P1 and

P4 can be selected as good producers of some of the essential oil components as carvone and limonene, respectively. The genotypes developed in the current study possess various superior agronomical traits of high commercial value. These improved genotypes can be adopted at a commercial scale to generate significant economic benefits. Chromosome doubling may play a crucial role in the breeding of *M. spicata* for further improvements.

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6. Synthetic polyploid induction influences morphological, physiological, and photosynthetic characteristics in *Melissa officinalis* L.

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This chapter explores the potential of oryzalin mediated in vitro polyploidization in inducing autopolyploid in Melissa officinalis L. and assesses its impact on the morpho physicochemical properties with special emphasis on essential oils.

Author contribution: Rohit Bharati was involved in conceptualization, data curation, investigation, methodology, software, writing -original draft, writing – review & editing.

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Abstract:

Melissa officinalis L., a well-known herb with diverse industrial and ethnopharmacological properties. Although, there has been a significant lack in the breeding attempts of this invaluable herb. This study aimed to enhance the agronomical traits of *M. officinalis* through *in vitro* polyploidization. Nodal segments were micropropagated and subjected to oryzalin treatment at concentrations of 20, 40, and 60 µM for 24 and 48 hours. Flow cytometry, chromosome counting, and stomatal characteristics were employed to confirm the ploidy level of the surviving plants. The survival rate of the treated explants decreased exponentially with increasing oryzalin concentration and duration. The highest polyploid induction rate (8%) was achieved with 40 µM oryzalin treatment for 24 hours. The induced tetraploid plants exhibited vigorous growth, characterized by longer shoots, larger leaves, and a higher leaf count. Chlorophyll content and fluorescence parameters elucidated disparities in photosynthetic performance between diploid and tetraploid genotypes. Tetraploid plants demonstrated a 75% increase in average essential oil yield, attributed to the significantly larger size of peltate trichomes. Analysis of essential oil composition in diploid and tetraploid plants indicated the presence of three major components: geranial, neral, and citronellal. While citronellal remained consistent, geranial and neral increased by 11.06% and 9.49%, respectively, in the tetraploid population. This effective methodology, utilizing oryzalin as an anti-mitotic agent for polyploid induction in *M. officinalis*, resulted in a polyploid genotype with superior morpho-physiological traits. The polyploid lemon balm generated through this method has the potential to meet commercial demands and contribute significantly to the improvement of lemon balm cultivation.

Keywords: chromosome doubling, crop improvement, essential oil, *Melissa officinalis*, oryzalin, polyploid induction, polyploidization

6.1 Introduction

Melissa officinalis, commonly known as lemon balm, is a perennial crop belonging to the Lamiaceae family. It is widely cultivated for culinary uses, herbal tea production, and essential oil extraction owing to its aromatic leaves with a lemony scent and flavor (Dastmalchi et al., 2008; Shakeri et al., 2016). Additionally, lemon balm possesses numerous medicinal properties. For instance, it has been used to remedy conditions such as gastrointestinal disease, rheumatoid arthritis, or neurological disorders in traditional Asian medicines (Shakeri et al., 2016; Miraj et al., 2017). In folk medicines of several European countries, it is used for treating insomnia, sore throat, cough, nervousness, hepatic, and biliary ailments (Shakeri et al., 2016). Several recent studies have also demonstrated lemon balm to possess antioxidant, hypolipidemic, anti-cancer, anxiolytic, antimicrobial, and anti-inflammatory effects (Shakeri et al., 2016; Miraj et al., 2017). These pharmacological effects are primarily attributed to the various phytochemicals present in the essential oils of lemon balm. The essential oils of *M. officinalis* also contain several citral isomers, largely geranial, and neral, providing the plant with its characteristic lemon-scented aroma (Shakeri et al., 2016).

Plant breeding plays a pivotal role in enhancing the genetic diversity and adaptability of plants. However, in the case of *M. officinalis*, breeding attempts have been conspicuously scarce. This lack of attention provides an intriguing opportunity to delve into the unexplored capabilities of this crop. While traditional breeding approaches like hybridization, mass selection, and selective breeding have been successful, they are often time-consuming and cost-intensive (Salma et al., 2017). However, there are faster and cost-effective approaches like synthetic polyploidization for developing new genotypes with superior traits. Synthetic polyploidization, where anti-mitotic agents are employed to produce polyploid genotypes of an existing one, is gaining popularity among researchers for its numerous advantages (Dhooghe et al., 2011; Gantait and Mukherjee, 2021; Bharati et al., 2023b).

The polyploids generated through polyploidization often display superior agronomical traits and outperform their progenitor genotype (Niazian and Nalousi, 2020; Gantait and Mukherjee, 2021). For instance, leaf area increased significantly in *Thymus vulgaris* by 10.4% after polyploidization, whereas in *Anemone sylvestris,* a remarkable increase of 122.23% in the leaf length was observed (Zahumenická et al., 2018; Homaidan Shmeit et al., 2020). Owing to this potency of polyploidization, researchers have also successfully employed this technique to enhance the essential oil yields across various medicinal and aromatic plants. Some examples include an increased amount of essential oils in *Thymus vulgaris* (Homaidan Shmeit et al.,

2020), *Lippia integrifolia* (Iannicelli et al., 2016), *Mentha* × *villosa* (Moetamedipoor et al., 2022), *Mentha spicata* (Bharati et al., 2023a), and *Zingiber officinale* Roscoe (Zhou et al., 2020). Induced polyploid plants often display higher chlorophyll contents with an enhanced photosynthetic capacity (Cao et al., 2018; García-García et al., 2020; Ulum et al., 2021).

Considering these developments, it could be hypothesized that polyploidization could be an excellent breeding approach to trait improvement in *M. officinalis*. Previously, colchicine has been predominantly used to induce polyploidy across a wide range of plant species due to its thermostability and effectiveness (Dhooghe et al., 2011; Eng and Ho, 2019). However, colchicine has multiple reported side effects, such as abnormal growth, sterility, and gene mutation, and it can lead to toxicity to humans (Dhooghe et al., 2011; Beranová et al., 2022). As an alternative, mitosis-inhibiting herbicides, including oryzalin, are becoming popular among researchers. Given that oryzalin has a higher affinity towards plant tubulin, it is more effective and provides better results when compared with colchicine at much lower concentrations (Ebrahimzadeh et al., 2018; Niazian and Nalousi, 2020). The application of anti-mitotic compounds to plants can be carried out either in *ex-vitro* or *in vitro* conditions. While *ex-vitro* conditions are more convenient, *in vitro* conditions offer better control and effectiveness, making them the preferred option for inducing polyploidy (Bharati et al., 2023a).

The objective of the study was to induce polyploidy in *M. officinalis* using oryzalin as an anti-mitotic agent under *in vitro* conditions. The current study also aims to assess the potential impact of polyploidization towards the enhancement of agronomical traits, including essential oil yield and anatomical, morphological, and photosynthetic attributes in lemon balm. The findings of this study will form a basis for breeding attempts in lemon balm and other medicinal and aromatic herbs. The developed protocol can be adopted at a commercial scale to generate novel genotypes with superior characteristics of various medicinal and aromatic plants.

6.2 Materials and Methods

6.2.1 Procurement of plant materials and *in vitro* establishment

Plant samples of *M. officinalis* (2n=2x=32) were obtained from the maintained medicinal plant collection at the botanical garden of the Faculty of Tropical Agrisciences, Czech University of Life Sciences Prague, Czech Republic. *M. officinalis* obtained was diploid with 16 basic number of chromosomes (x = 16) (Kittler et al., 2015). These samples were held in a greenhouse with an average temperature of 23 °C and a relative humidity of 70-80%. Stems with a minimum of 5 nodes were carefully selected and washed with running tap water for 10 minutes. Nodal segments of approximately 1 cm were then cut and subjected to surface sterilization. The sterilization began by treating the explants with 70% ethanol (*v/v*) for 2 minutes. Subsequently, the explants were immersed and continuously stirred in a 1% (*v/v*) solution of sodium hypochlorite (commercial bleach-SAVO, NaClO) containing two drops of Tween-20 for 10 minutes. Afterward, the explants were rinsed three times using sterile double distilled water. The treated explants were then transferred to a laminar airflow hood for inoculation. The sterilized nodal segments were placed in 250 mL Erlenmeyer flasks containing approximately 50 mL of Murashige and Skoog's basal medium (Murashige and Skoog, 1962). The medium was supplemented with 3% sucrose and 0.8% agar, and the pH was adjusted to 5.7 ± 0.1. The inoculated cultures were maintained in a growth room with a temperature of 24/20±1 °C and relative humidity between 65-70%. The photoperiod was set to 16/8 hours (light/dark) using white, fluorescent lamps with an intensity of 3800 lux (51.3 µmol m^{-2} s^{-1}). The plants were subcultured every 14 days until a sufficient number of plants were obtained for polyploid induction.

6.2.2 Anti-mitotic agent treatment

Nodal segments of approximately 1-1.5 cm were placed in beakers containing MS medium and left for 48 hours before the treatments were applied. Oryzalin (Sigma-Aldrich, Prague, CZ) was dissolved in 1% Dimethyl sulfoxide (DMSO) to create three oryzalin solutions of different concentrations (20, 40, and 60 µM). These oryzalin solutions were then carefully poured onto the explants and maintained for 48 hours in a sterile laminar flow box until all the explants were submerged. The experiment consisted of six treatments labeled T1 to T6, which involved using oryzalin at different concentrations and exposure durations. The specific treatments were as follows: T1 (20 µM for 24 hours), T2 (20 µM for 48 hours), T3 (40 µM for 24 hours), T4 (40 µM for 48 hours), T5 (60 µM for 24 hours), and T6 (60 µM for 48 hours). A control variant

(Control) was also used, where explants were subjected to MS medium without any oryzalin treatment. A total of 50 nodal segment explants were used for each treatment. After the treatment period, all the treated samples were taken out of the oryzalin solutions and rinsed three times with sterile double-distilled water. These washed samples were then placed back onto the MS medium and maintained for six months, with regular subculturing every 30 days. Every three months, the ploidy level of the samples was determined using a flow cytometer (Partec GmbH in Münster, Germany).

6.2.3 Transfer to the *ex-vitro* conditions

The control and the identified polyploid plants were transferred in a greenhouse with an average temperature of 23 °C and relative humidity between 60-70%. The plants were subjected to primary and secondary hardening. Primary hardening mainly involved transferring the plants in a pot (5×5 cm) containing garden soil and perlite (3:1 ratio) under polythene covering for seven days. Following this, the polythene covering was removed, and the plants were subjected to an additional seven days under greenhouse conditions. Subsequently, the plants were transferred to field conditions (50°07'51.1"N 14°22'15.2"E) for a 60-day period, during which morphophysiological data and plant samples were collected for further analysis.

6.2.4 Morphological parameters

Morphological data were collected from the control diploid and induced polyploid plants to assess the influence of polyploidization. The evaluated parameters included: the number of shoots, shoot length, number of nodes per shoot, internodal distance, number of leaves per shoot, leaf area, leaf thickness, stem thickness, and wet weight. Data were also collected for floral and seed characteristics. A total of 20 observations were taken for all the attributes, excluding seed weight, which was determined by measuring the weight of 100 seeds across 10 replicates.

6.2.5 Stomatal observation

The length and width of stomata, along with the stomatal density from diploid and tetraploid plants, were compared using the nail polish impression method previously described (Grant and Vatnick, 2004; Parsons et al., 2019). The abaxial surface of the leaves was coated with clear nail polish and allowed to dry. Subsequently, imprints were obtained using transparent tape and placed on a glass slide to measure the size and density of stomata under a light microscope at 60× and 100× magnification.

6.2.6 Flow cytometry analysis

Flow cytometric analyses were performed as described by Doležel et al. (2007) with slight modifications. Briefly, a leaf section of approximately 1cm^2 was placed in a petri dish containing 1 mL of Otto I buffer (0.1 M $C_6H_8O_7$, 0.5% Tween 20) and chopped using a razor blade. The crude suspension was then filtered through a nylon mesh (50 µm), and the filtrate was collected. After that, 1 mL of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_2O$) was added to the filtrate containing 2 µg/mL of DAPI (fluorescent dye). The prepared samples were analyzed through a Partec PAS flow cytometer (Partec GmbH, Münster, DE), where a minimum of 10000 nuclei per sample were measured. The relative content of DNA was recorded in the form of a histogram using the Flomax software package (Version 2.3).

6.2.7 Chromosomes counting

Chromosome counting was performed in control and induced polyploid plants following a previously published method (Bharati et al., 2023a) with slight adjustments. Approximately 1 cm long fresh root tips were collected in the early morning between 7 and 8 o'clock and immersed in a saturated solution of Paradichlorobenzene at room temperature for 2 hours. Subsequently, the root tips were rinsed three times with distilled water and submerged in a freshly prepared solution of ethyl alcohol and acetic acid (3:1) (*v/v*) for 60 minutes at room temperature. After removing the roots from the solution, they were washed thrice with distilled water before undergoing the hydrolysis and staining steps. The root tips were then subjected to a 10-minute incubation in a 1 N HCl solution at 60 °C, followed by staining with Schiff's reagent for 1 hour. Finally, the root tips were further trimmed to approximately 0.2 cm and placed on a glass slide with a drop of 2% orcein-acetic solution for visualization under a BX51 Olympus light microscope (Olympus Optical Co., Tokyo, Japan) at a magnification of 100×. For each genotype, a minimum of 5 root tips were randomly selected and chromosomes were counted across 10 microscopic fields per root tip to ensure accurate representation.

6.2.8 Non-destructive and destructive chlorophyll estimation

Non-destructive chlorophyll estimation was carried out using a SPAD-502, Minolta Camera CO, Japan, to compare the relative chlorophyll content between the diploid and tetraploid genotypes. A total of 20 measurements from each genotype were taken. To further validate the SPAD values, a previously established destructive method (Mosa et al., 2018) with slight modifications was used to estimate chlorophyll levels among the diploid and tetraploid

genotypes. Fresh leaf samples were obtained from control diploid and induced tetraploid plants and were crushed into a fine powder using liquid nitrogen and a mortar and pestle. Accurately, 300 mg of the powdered sample was mixed with 5 mL of 80% acetone (*v/v*) and shaken in the dark for 15 minutes. Afterward, the mixture was centrifuged for 15 minutes at 4 °C and 3000 rpm. The resulting liquid (supernatant) was collected and diluted with 80% acetone (*v/v*) in a 1:5 ratio. Finally, the absorbance of chlorophyll a (663 nm) and chlorophyll b (645 nm) was measured using a spectrophotometer. The total chlorophyll content in the diploid and tetraploid plants were calculated as previously described by Manzoor et al. (2018). Destructive chlorophyll estimation was carried out in three replicates.

6.2.9 ICP-MS analysis of micro and macronutrients

Approximately 0.2 g of dried samples from control and polyploid genotypes were measured and placed in a quartz vessel. Then, 2 mL of H_2O_2 (Rotipuran®, Carl Roth, Germany) and 4 mL of HNO_3 (Analpure®, Analytika, Czech Republic) were added to the samples. The prepared samples were digested in a closed vessel microwave system at 180 °C for 20 mins. After digestion, the solutions were transferred to 50 mL polypropylene tubes and filled with Milli-Q water (≥ 18.2 MΩ cm^{-1}; MilliQ system, Millipore, SAS, France) to reach a final volume of 45 mL. The elemental concentration of certain macro and micro elements was then examined using inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7700×, Agilent Technologies Inc., USA). To ensure quality, a certified reference material, specifically Peach leaves (SRM-1547, NIST), was used. For each genotype, three biological and three technical replicates were examined.

6.2.10 Comparison of Chlorophyll fluorescence kinetics

To assess the photosynthetic performance of the diploid and tetraploid genotypes, we employed a portable FluorPen FP110 (manufactured by PSI, Czech Republic) and a MultispeQ V 2.0 device connected to the PhotosynQ platform (www.photosynq.org) with their default settings. These measurements were conducted on diploid and tetraploid plants under field conditions, with data collected at three different times of the day: morning (8:00 AM), afternoon (1:00 PM), and evening (5:00 PM). For FluorPen, the intact leaves were dark-adapted using leaf clips for 30 minutes. All the measured and calculated parameters from both the portable chlorophyll fluorometer included: LEF- linear Electron Flow, gH+ - proton conductivity of the thylakoid membrane, NPQt- Non-Photochemical Quenching, Phi2- Quantum Yield of Light-Adapted Photosystem II (PSII) Electron Transport, Fv/Fm -maximum quantum yield of PSII

photochemistry, PIabs - absorption flux per active reaction center, ABS/RC - absorption per active reaction center. For the derivations and definitions of these parameters, one can refer to Stirbet and Govindjee (2011), Banks, (2017), and Kuhlgert et al. (2016).

6.2.11 Essential oil extraction and GC/MS analysis

Fresh aerial parts of diploid control and tetraploid plants were collected after 60 days of cultivation and dried until a constant dry weight was achieved in a drying oven (UF55plus, Memmert, USA) at 30 °C. The dried samples were then subjected to hydro-distillation using a Clevenger-type apparatus in triplicate for two hours. The extracted oils were subsequently dried over anhydrous sodium sulfate and stored in airtight glass vials at 4 °C until they were used for Gas chromatography-mass spectrometry (GC/MS) analysis. To identify the components of the essential oil, an Agilent 7890A gas chromatograph (GC) coupled with an Agilent 5975C single-quadrupole mass detector was utilized. The quantification was performed using an Agilent 6890A GC with a flame ionization detector (FID). Both instruments were equipped with an HP-5MS (30 m x 250 μm x 0.25 μm) non-polar column (Agilent, Santa Clara, CA, USA). One μL of sample diluted 1:1000 in hexane (VWR, Stribrna Skalice, Czech Republic) was injected in the split ratio of 12:1 into the inlet heated to 250 °C. The initial oven temperature was set to 60 °C for 3 min, then it was gradually increased at the rate of 5 °C/min up to 231 °C and kept constant for 10 min. The FID detector was heated to 250 °C. Helium was used as a carrier gas at the flow rate of 1 mL/min. The MS analysis was carried out in full scan mode with the electron ionization energy set to 70 eV. The relative percentage content was expressed as the ratio of individual peak area to the total area of all peaks. The identification of the essential oil components was based on a comparison of mass spectra and Kovat's retention indices with those in the National Institute of Standards and Technology Library (NIST, USA) as well as in the literature (Adams, 2007). The identification of 9 components were confirmed using authentic standards. All the standards were obtained from Sigma–Aldrich (Prague, Czech Republic).

6.2.12 Statistical analysis

The data from the morphological, biochemical, and anatomical parameters of diploid and induced polyploid genotypes were subjected to statistical analysis using Student's t-test. All statistical tests were conducted with a significance level set at $p < 0.05$ using the Microsoft Excel 2021 software package. The graphs presented in the study were generated using

GraphPad Prism (Version 9.4.0). Microscopic images were measured and analyzed using Image J software (IJ 1.46r).

6.3 Results

6.3.1 Effect of oryzalin on survival and polyploid induction rate

Out of the 300 nodal segments treated to various concentrations and duration of oryzalin, 113 plants survived. The survival rate of the plants decreased significantly with the increasing concentration of oryzalin (Table 6.1). The highest and the lowest survival rate were observed in T1 (54%) and T6 (24%), respectively (Table 6.1). The histograms generated through the flow cytometric analysis were effective in distinguishing the induced polyploids from the diploid (Figure 6.1A,B). A total of 8 polyploids were identified among the surviving population. The polyploid induction rate was highest in T3 (8%), while T4, T5, and T6 exhibited lower effectiveness, resulting in yields of just 4%, 2%, and 2%, respectively. The treatments T1 and T2 were found ineffective, with a polyploid induction rate of 0%. The ploidy of the control and polyploid genotypes were also validated through chromosome counting. It was affirmed that the chromosome numbers in the treated plants were doubled ($2n = 4x = 64$) compared with the diploid genotype ($2n = 2x = 32$) (Figure 6.1C,D).

Table 6.1 Polyploidization and survival rate of nodal segments of *M. officinalis* treated with oryzalin.

Treatment	Oryzalin (µM)	Number of treated explants	Time of treatment (h)	Survival rate (%)	Polyploid induction rate (%)
Control	0	50	n.a	100±0.0	n.a
T1	40	50	24	54±6.5a	0.0±0.0
T2	40	50	48	44±6.3b	0.0±0.0
T3	60	50	24	36±5.9c	8.00±0.77a
T4	60	50	48	36±5.9c	4.00±0.42b
T5	80	50	24	32.5±5.4c	2.00±0.48c
T6	80	50	48	24±6.5d	2.00±0.48c

Different superscript letters within the same column differ significantly (Student's t-test, $p < 0.05\%$). 'n.a' indicates not applicable.

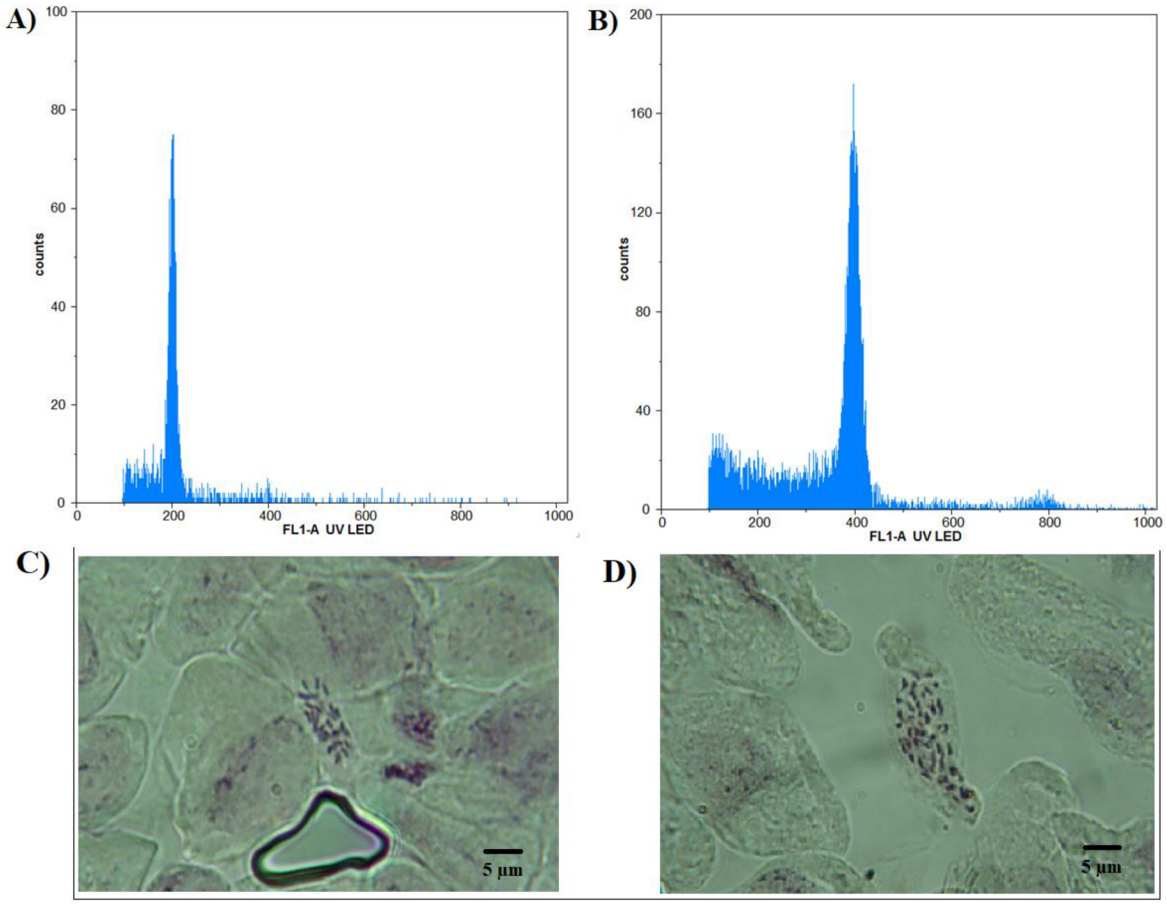

Figure 6.1 Histogram obtained from flow cytometry analysis for (A) diploid and (B) tetraploid plant, depicting relative DNA content along with chromosomes under 100x magnification for (C) diploid and (D) tetraploid plant.

6.3.2 Morpho-physiological characteristics

Significant differences were observed in both the morphological and physiological characteristics of diploid and tetraploid plants outlined in Table 6.2. Tetraploid plants displayed a noteworthy increase in average shoot length and the number of nodes per shoot, with respective increments of 34.51% and 21.23% compared with the diploid control genotypes. Conversely, the average number of shoots per plant decreased in the tetraploid population, while internodal distance remained unaffected. Notably, leaf area, leaf thickness, and the number of leaves per shoot were positively impacted in tetraploid plants, exhibiting remarkable increases of 18.78%, 45.44%, and 52.63%, respectively, compared with the diploid plants (Figure 6.2) (Table 6.2). Furthermore, a significant rise in average stem thickness was observed, elevating from 2.8 mm in diploid plants to 3.48 mm in tetraploid plants. The average fresh biomass per plant also experienced an upward trend, reaching 101.76±7.82 g in tetraploids, as opposed to 85.3±6.08 g in diploid plants (Table 6.2). Floral characteristics also displayed superior attributes

in tetraploids. Specifically, the average length of the flower, width of the calyx, and width of the corolla exhibited an upward trend in tetraploid plants, with respective increases of 10.90%, 55.30%, and 30.04%. However, the length of the calyx remained unaffected (Figure 6.3) (Table 6.3). The average seed weight exhibited a significant increase in tetraploid plants, reaching 108.6±3.8 mg for every 100 seeds, compared to 73.15±2.48 mg in diploid plants. Likewise, the average seed width in the tetraploid population expanded to 12.01±1.04 mm from the 9.14±0.62 mm observed in diploid plants. However, the average seed length did not display a significant difference (Figure 6.4) (Table 6.3).

Figure 6.2 Comparison of leaf morphology from mother diploid and induced tetraploid plant.

Table 6.2 Morpho-physiological characteristics of diploid control and tetraploid genotypes of *M. officinalis.*

Variant	Number of shoots	Shoot length (cm)	Number of nodes per shoot	Internodal distance (cm)	Number of leaves per shoot	Leaf area (cm^2)	Leaf thickness (mm)	Stem thickness (mm)	Wet weight (g)
Diploid	24.28±1.79a	19.96±3.81b	5.98±0.86b	4.11±1.29a	11.98±1.95b	26.54±3.88b	0.38±0.05b	2.8±0.41b	85.3±6.08b
Tetraploid	21.76±2.07b	26.85±1.24a	7.25±1.02a	3.32±0.63a	14.23±1.84a	38.6±2.41a	0.58±0.08a	3.48±0.53a	101.76±7.82a

Different superscript letters within the same column differ significantly (Student's t-test, $p < 0.05\%$) (n=20).

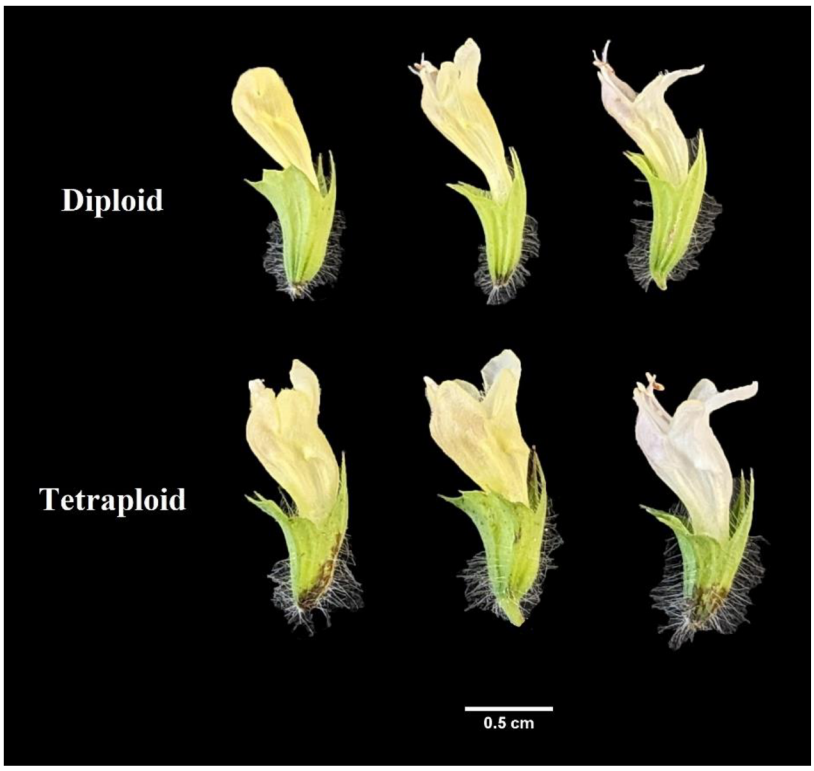

Figure 6.3 Comparison of floral characteristics between (A) mother diploid and (B) induced tetraploid plant.

Table 6.3 Flower and seed characteristics of diploid and induced tetraploid genotypes of *M. officinalis*.

Variant	Length of flower (mm)	Length of calyx (mm)	Width of calyx (mm)	Width of corolla (mm)	Average seed length (mm)	Average seed width (mm)	Average seed weight of 100 seeds (mg)
Diploid	13.57±0.62b	7.18±0.94a	3.49±0.02b	4.76±0.28b	19.05±0.57a	9.14±0.62b	73.18±2.48b
Tetraploid	15.05±0.46a	7.7±1.21a	5.42±1.22a	6.19±0.32a	19.35±0.53a	12.01±1.04a	108.06±3.8b

Different superscript letters within the same column differ significantly (Student's t-test, $p < 0.05\%$) (n=20).

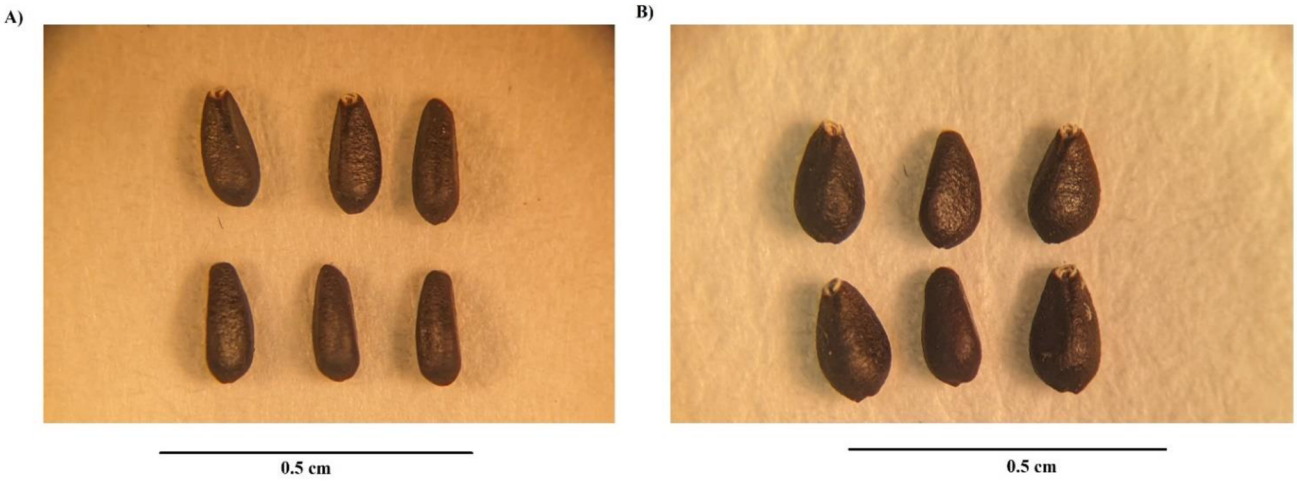

Figure 6.4 Morphology and structure comparison of seeds from (A) control diploid and (B) induced tetraploid plants of *M. officinalis*.

6.3.3 Anatomical comparison

Notable differences in the anatomical characteristics between the tetraploid and diploid genotypes were observed. The stomatal frequency decreased significantly in the tetraploid population, although the average length and width of the stomata increased from 11.34±1.95 µm and 7.86±1.05 µm in diploid to 20.76±1.52 µm and 12.57±0.79 µm in tetraploid plans, respectively (Table 6.4) (Figure 6.5A,B,C,D). Furthermore, the length of the guard cells were positively influenced and increased from 16.21±2.34 in diploid to 25.72±2.23 µm tetraploid plants. Additionally, the peltate trichomes density, which is chiefly responsible for the oil secretion, was also influenced, and the average diameter of oil-secreting glands increased from 20.85±1.61 µm in diploid to 30.24±2.18 in the tetraploid population (Figure 6.6A-D).

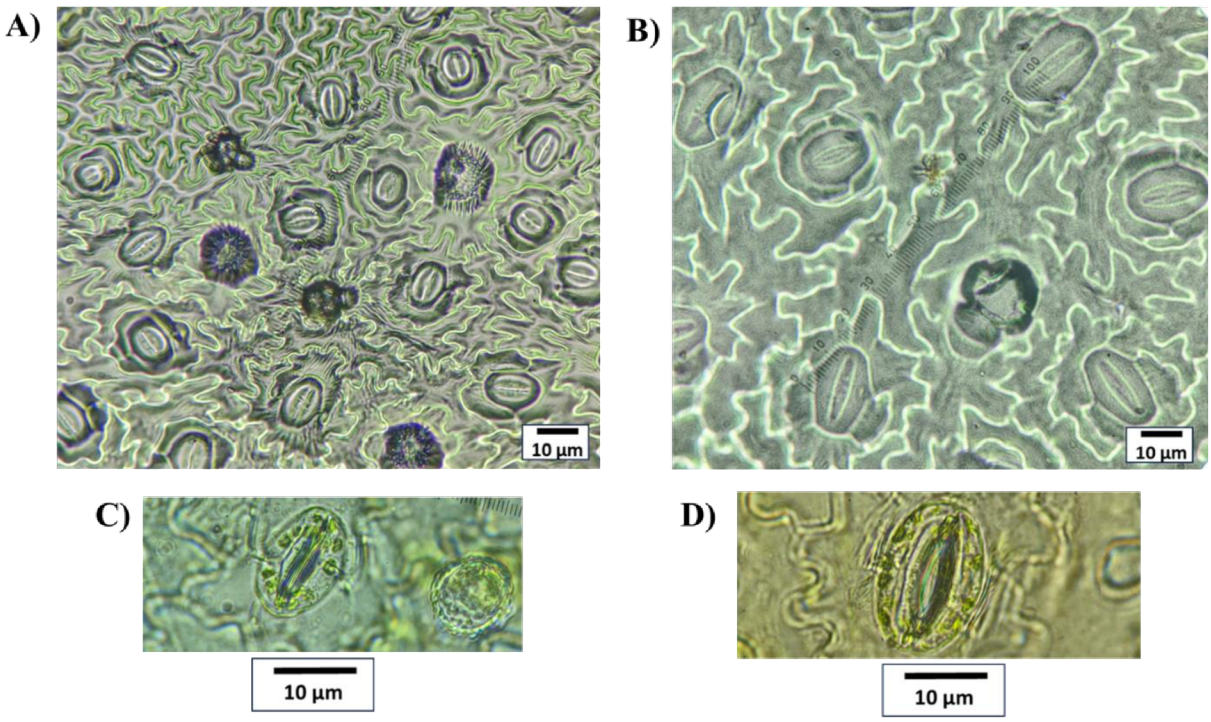

Figure 6.5 Stomata size and frequency comparison between (A) control diploid and (B) induced tetraploid plant. The images (A) and (B) were captured under 60× and (C) and (D) under 100× magnification.

Table 6.4 Comparison of stomatal characteristics between diploid and induced polyploid genotypes of *M. officinalis*.

Variant	Average number of stomata per magnification field (60x)	Average stomata length (µm)	Average stomata width (µm)	Average guard cell length (µm)
Diploid	24.33±3.21a	11.34±1.95b	7.86±1.05b	16.21±2.34b
Tetraploid	10.00±1.50b	20.76±1.52a	12.57±0.79a	25.72±2.23a

Different superscript letters within the same column differ significantly (Student's t-test, $p < 0.05\%$) (n=20).

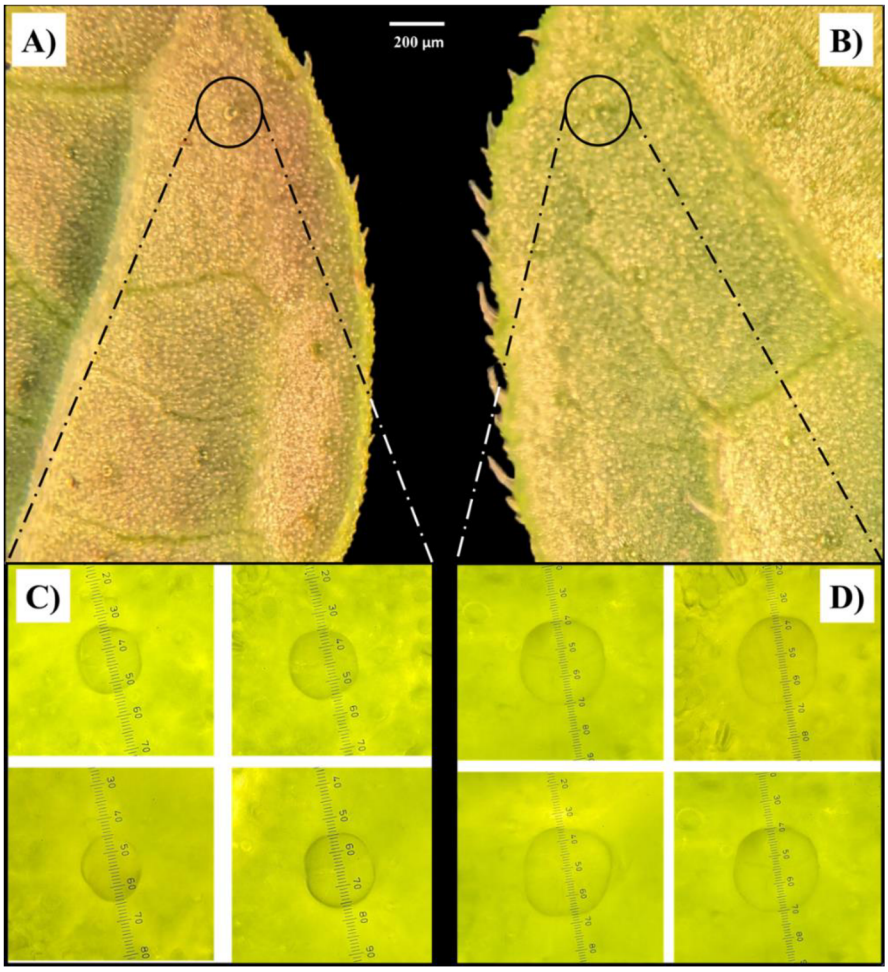

Figure 6.6 Comparison of peltate trichome gland sizes between (A, C) diploid and (B, D) induced tetraploid plants. The images were captured under 10× magnification (A, B) and 40× magnification (C, D). The measurements in C and D are presented in micrometers (µm).

6.3.4 Chlorophyll content

The average SPAD values increased significantly rising from 26.20±3.91 in diploid to 36.39±5.07 in tetraploid plants (Figure 6.7A). Similarly, destructive estimation of chlorophyll content revealed a significant increase from 0.93±0.05 mg/g FW in diploid plants to 1.32±0.07 mg/g FW in tetraploid plants (Figure 6.7B).

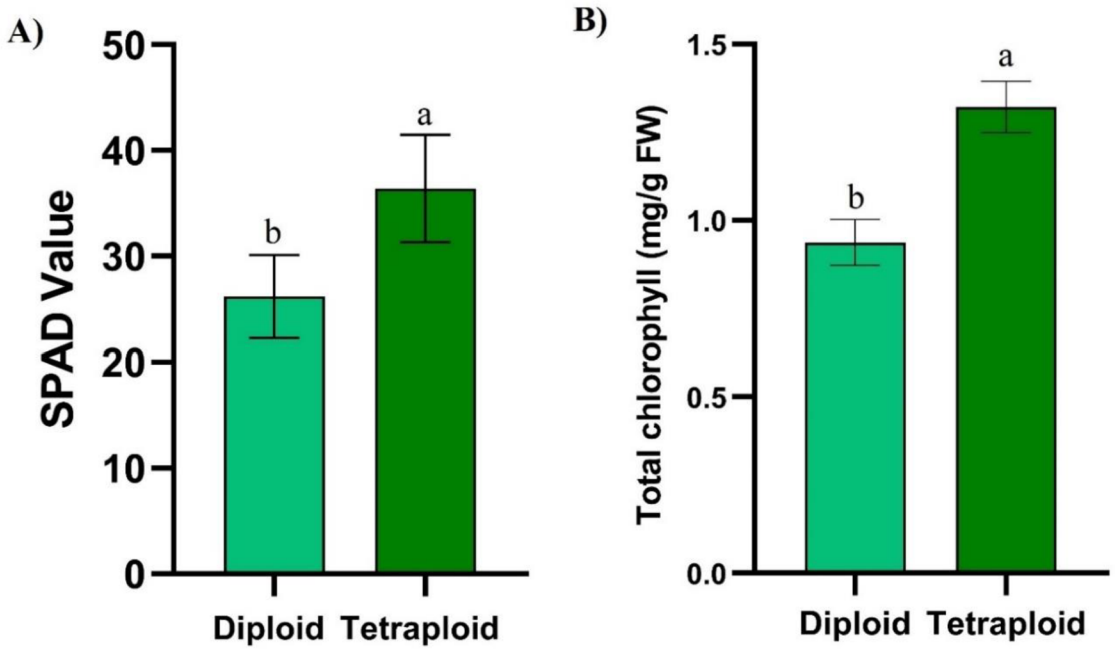

Figure 6.7 Graphs depicting (A) SPAD values and (B) Total chlorophyll content comparison between control diploid and induced tetraploid plant. Different superscript letters on the vertical bars differ significantly ($p < 0.05$).

6.3.5 Chlorophyll fluorescence kinetics

In our comparative study of diploid and induced tetraploid genotypes of *M. officinalis*, we observed that Fv/Fm, NPQt, and Phi2 values exhibited no significant differences between the two genotypes throughout the day (Figure 6.8A,F,G). Conversely, PIabs and ABS/RC values displayed consistent and statistically significant distinctions between diploid and tetraploid plants across all time points (morning, afternoon, and evening) (Figure 6.8B,C). For PIabs, diploid values ranged from 1.3 to 3.31, while tetraploid values were consistently higher, ranging from 2.88 to 4.48. ABS/RC values also exhibited significant differences, reflecting variations in absorption per active reaction center between the two genotypes. In the diploid genotype, ABS/RC values ranged from 1.79 to 2.03, whereas in the tetraploid genotype, values ranged from 1.54 to 1.82. Linear Electron Flow (LEF) showed a significant increase in the tetraploid genotype in the morning and afternoon but became statistically insignificant in the evening. In the morning, LEF was 7.19 for diploid and 23.52 for tetraploid, while in the afternoon, it was 14.0 for diploid and 31.635 for tetraploid (Figure 6.8D). Proton conductivity of the thylakoid membrane (gH+) displayed a significant difference between the genotypes only in the afternoon, with values of 116.71 for diploid and 171.82 for tetraploid (Figure 6.8E). These results underscore the genotype-specific variations in photosynthetic parameters, particularly

PIabs and ABS/RC, and their dependency on the time of day, suggesting intricate regulatory mechanisms at play in response to changing environmental conditions.

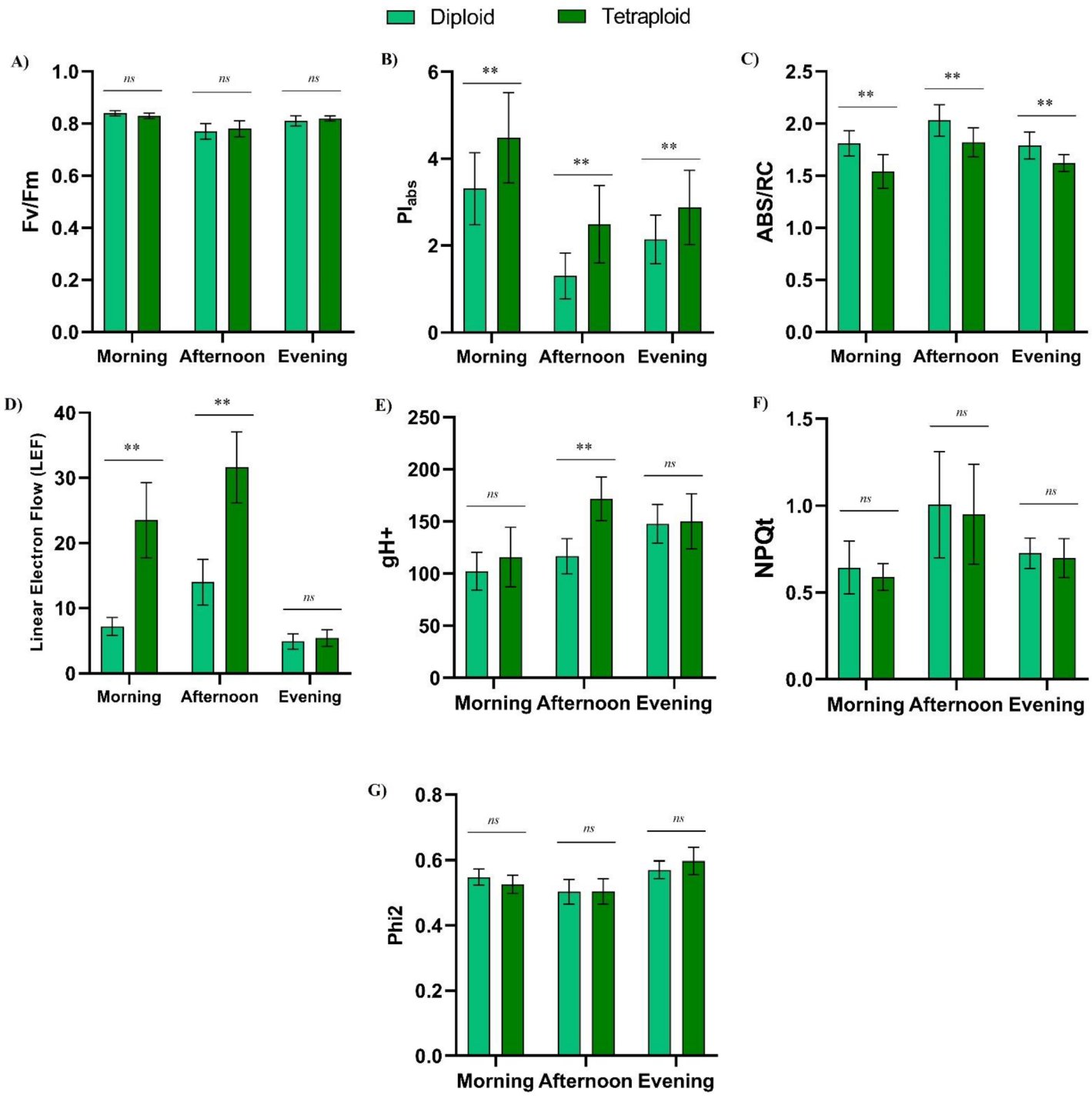

Figure 6.8 Comparison of diurnal fluctuations in chlorophyll fluorescence metrics (A) Fv/Fm, (B) PIabs, (C) ABS/RC, (D) linear electron flow (LEF), (E) gH+, (F) NPQt, and (G) Phi2 between control diploid and induced tetraploid plants. '**' signifies significant difference at $p<0.5$.

6.3.6 Macro- and micronutrients content

The results of the ICP-MS analysis comparing diploid and tetraploid genotypes of *M. officinalis* revealed distinct changes in the elemental composition. The potassium concentration increased significantly while the calcium concentrations decreased in the induced polyploids. Phosphorus, ferrous, and boron concentrations were unaffected by chromosome doubling and remained same between the two genotypes. On the other hand, copper, manganese, and zinc concentrations were observed to decrease in the tetraploid genotype compared with the diploid genotype (Table 6.5).

Table 6.5 Macronutrient and micronutrient analyzed from leaves of diploid and induced polyploids of *M. officinalis* plants (mg/kg).

Variant	K	Ca	P	Fe	Cu	Zn	B	Mg
Diploid	27867.99 ±911.83b	11755.70 ±302.79a	4159.51± 20.59a	120.69 ±10.78a	14.53 ±0.33a	49.09 ±3.42a	23.87 ±0.82 a	5294.86± 54.97a
Tetraploid	34438.93 ±249.30a	8019.07± 441.131b	3651.71± 227.62a	100.71 ±6.07a	11.73 ±0.61 b	31.50 ±2.75 b	24.14 ±0.84 a	4450.96± 134.95b

Different superscript letters within the same column differ significantly (Student's t-test, $p < 0.05$) (n=20).

6.3.7 Essential oil yield and composition

The essential oil yields were 0.21% (*v/w*) in tetraploid plants, whereas the diploid plants yielded only 0.12% (*v/w*) of essential oil. A total of 21 components were identified, which represents 90.97% and 92.49% of the total composition of the oils from diploids and tetraploid, respectively (Table 6.6). The major components were geranial, neral, and citronellal, constituting 73.23% and 80.10% of the total composition in diploids and tetraploids, respectively. The remaining components didn´t exceed 5%. Although, two of the significant minor components, geranyl acetate and caryophyllene oxide reduced and were 4.11 and 4.23% in diploid and 3.42 & 2.68% in tetraploid, respectively. Among the major components, neral and geranial increased significantly, rising from 27.60% and 30.22% in diploid to 39.23% and 43.57% in the tetraploid genotype, respectively. On the other hand, the other major component, citronellal, was unaffected by chromosome doubling (Table 6.6).

Table 6.6 Composition of diploid and tetraploid *Melissa officinalis* essential oils

RI [a]	***RI(lit)*** [b]	**Compound**	**Content (%) ± SD** [c]		**Identification**[d]
			Diploid	**Tetraploid**	
700	700	Heptane	0.21±0.008	0.07±0.002	MS, RI, Std
800	800	Hexanal	0.32±0.004	0.08±0.002	MS, RI
852	854	*E*-2-Hexenal	0.44±0.001	0.32±0.001	MS, RI
980	980	β-Pinene	0.87±0.003	0.47±0.001	MS, RI, Std
1057	1055	Benzeneacetaldehyde	0.50±0.012	0.34±0.004	MS, RI
1099	1098	Linalool	0.35±0.010	0.51±0.001	MS, RI, Std
1147	1147	7-Methyl-3-methyleneoct-6-enal	0.48±0.006	0.39±0.008	MS, RI
1156	1153	**Citronellal**	**6.40±0.013**	**6.31±0.012**	MS, RI, Std
1167	1165	Isoneral	0.20±0.006	0.45±0.006	MS, RI
1184	1184	3,7-Dimethyl-3,6-octadienal	1.03±0.026	0.72±0.010	MS, RI
1244	1240	**Neral**	**27.60±0.017**	**30.22±0.016**	MS, RI, Std
1257	1255	Geraniol	0.06±0.002	0.85±0.002	MS, RI, Std
1264	1261	Methyl citronellate	1.29±0.004	0.51±0.001	MS, RI
1273	1270	**Geranial**	**39.23±0.040**	**43.57±0.022**	MS, RI, Std
1325	1323	Methyl geraniate	0.70±0.002	0.31±0.003	MS, RI
1385	1383	Geranyl acetate	4.11±0.007	3.42±0.003	MS, RI
1419	1418	β-Caryophyllene	0.00±0.000	0.17±0.005	MS, RI, Std
1430	1436	α-Bergamotene	0.27±0.003	0.22±0.002	MS, RI
1490	1493	β-Ionone	1.75±0.016	0.38±0.001	MS, RI
1580	1574	Germacrene D-4-ol	0.93±0.023	0.49±0.011	MS, RI
1582	1581	Caryophyllene oxide	4.23±0.021	2.68±0.004	MS, RI, Std
		Total identified	90.97	92.49	

a: Kovat´s retention indices measured on HP-5MS column; b: retention indices from literature; c: relative percentage content based on total area of all peaks (average of three extractions); d: Identification based on comparison of mass spectra (MS) and retention indices (RI) with NIST library and literature; Std: identification confirmed by co-injection with authentic standard.

6.4 Discussion

Polyploidization attempts have been made in lemon balm using colchicine to obtain polyploid genotypes (Borgheei et al., 2010; Talei and Fotokian, 2020). Although, the previous studies failed to provide specific information on the concentration and duration effective for the induction of tetraploid plants in *M. officinalis*. On the other hand, oryzalin has never been assessed as an anti-mitotic agent in this species. In the current study, the use of oryzalin as an anti-mitotic agent successfully generated polyploids in *M. officinalis,* establishing the potency of oryzalin as an anti-mitotic agent in this plant species. The efficacy of oryzalin as an anti-mitotic agent has been well established. Oryzalin exhibits a higher affinity towards plant tubulin and lower toxicity than colchicine, making it a preferred choice for chromosome doubling in plants (Dhooghe et al., 2011; Niazian and Nalousi, 2020). For instance, a study by Sakhanokho et al. (2009), tested the efficacy of colchicine and oryzalin for inducing polyploids in Ornamental Ginger (*Hedychium muluense*), and oryzalin was found more effective with 15% induction frequency compared with colchicine with just 13%. Interestingly, the effective concentration for oryzalin was just 60 µM, whereas colchicine was 2.5 mM. Similar results where oryzalin performed better than colchicine have been reported in *Passiflora edulis* Sims. (Rêgo et al., 2011), *Watsonia lepida* (Ascough et al., 2008) and some of the *Vaccinium species* (Tsuda et al., 2013). It is worth noting that none of the treatments in our study resulted in mixoploidy, a phenomenon frequently observed in synthetic polyploidization. This suggests that *M. officinalis* may be inherently unstable in a mixoploid state and prefers to exist either as a diploid or a tetraploid when induced using oryzalin.

Morphological variations in the induced polyploid are a well-known phenomenon called the "gigas effect". The induced polyploids often exhibit enlarged organs compared with their progenitors. For instance, a significant increase in leaf area, width, and thickness was observed in the induced polyploids of *Thymus vulgaris* and *Agastache foeniculum* L. (Talebi et al., 2017; Homaidan Shmeit et al., 2020). Similarly, a recent study on polyploidization in *Mentha spicata* elucidated that the polyploids displayed vigorous growth with larger organs. Specifically, the polyploids of *M. spicata* observed an increase in leaf area, leaf, and stem thickness and nearly doubled compared with the control genotype (Bharati et al., 2023a). Comparable observations were observed in the current study where the tetraploid plants exhibited a more robust growth compared with the diploid plants, characterized by longer shoots, a higher number of nodes per shoot, larger leaves, and an increased leaf count per shoot. As a result, the tetraploid plants exhibited a notably bushier growth habit. Comparing the floral characteristics, it was evident

that the flower size increased significantly in the tetraploid compared with the diploid plants. Although, the floral traits were retained, such as petal number and flower color. Polyploidy-induced alterations in developmental processes may have influenced flower growth and differentiation, leading to changes in floral morphology. A similar increase in the size of the flower post-polyploidization has been reported across different plant species (Zahumenická et al., 2018; Bhattarai et al., 2021). The seed characteristics were influenced significantly where the tetraploid plants generated heavier and larger seeds. Similar increases in the seed size and weight in the polyploid genotypes have been reported in other plant species (Sadat Noori et al., 2017; Ding et al., 2023). The seed viability and the ploidy stability over generations need to be assessed and should be part of future studies.

Anatomical comparisons are frequently used as an indirect approach to assess the impact of polyploidization in plants. Stomatal observations were made to assess the difference between the stomata of the diploid and the induced tetraploid plants. The findings from the current study suggest that polyploidization affects the stomatal size significantly in this species, where it increased the length and width of the stomata by 83.06% and 59.92%, respectively, in the tetraploid population. A similar significant increase in stomatal size has been reported in lemon balm and other plant species from the Lamiaceae family due to chromosome doubling (Talei and Fotokian, 2020; Moetamedipoor et al., 2022; Bharati et al., 2023a). Although, with an increase in stomatal size, the density is often reported to decrease after polyploidization, which is consistent in the current study. The reduction in stomatal frequency is frequently linked to an increase in leaf epidermal cells, stomatal cells and a decrease in stomatal differentiation in induced polyploid plants (Shariat and Sefidkon, 2021).

Lemon balm subjected to polyploidization resulted in significantly higher chlorophyll content and similar increase in chlorophyll content in the induced polyploid plants is a well reported phenomenon (Feng et al., 2017; Münzbergová and Haisel, 2019; García-García et al., 2020). Further, the diurnal comparison of the chlorophyll fluorescence parameters provided valuable insights into the photosynthetic performance of the newly developed genotype across different time points of the day. Fv/Fm is a commonly used indicator of the maximum quantum yield of photosystem II (PSII), while NPQt and Phi2 are related to non-photochemical quenching and photosystem II quantum yield, respectively. These photosynthetic parameters showed no significant differences between the two genotypes throughout the day, indicating similar photosystem efficiency and photoprotective mechanisms (Stirbet and Govindjee, 2011), (Banks, 2017), and (Kuhlgert et al., 2016). However, PIabs and ABS/RC values consistently

differed, with the tetraploid genotype exhibiting higher PIabs and lower ABS/RC, that the tetraploid plants are more efficient in absorbing and utilizing light energy for photosynthesis (Kumar et al., 2020). Linear Electron Flow (LEF) was significantly higher in the tetraploid genotype in the morning and afternoon but leveled off in the evening. The tetraploid genotype's enhanced LEF in the morning and afternoon may indicate a more efficient conversion of absorbed light energy into electron transport, possibly due to higher pigment content or improved energy distribution within the thylakoid membranes (Kuhlgert et al., 2016). Proton conductivity (gH+) showed differences only in the afternoon, with higher values in the tetraploid genotype. This result implies that the tetraploid plants may have an enhanced capacity for proton transport across the thylakoid membrane during periods of increased photosynthetic activity, which could contribute to their improved photosynthetic performance in the afternoon (Avenson et al., 2005).

Trichomes are vital structures found in lemon balm, primarily responsible for secreting essential oils. Surprisingly, previous research on polyploidization in *M. officinalis* did not assess its impact on these oil-secreting glands known as peltate trichomes. Furthermore, the authors did not evaluate the essential oil yield in the induced polyploid plants (Borgheei et al., 2010; Talei and Fotokian, 2020). Conversely, the current study revealed a significant 45% increase in the size of these oil glands in tetraploid plants compared with diploid plants, indicating a corresponding enhancement in overall essential oil production in the tetraploid variants. Correspondingly, the essential oil extraction through hydro-distillation revealed a 75% increase in the tetraploid plants compared with the diploid plants. Enhanced levels of essential oils induced through polyploidization have been reported in various medicinal and aromatic plants from the Lamiaceae family. For instance, polyploidization in *Mentha×villosa* and *Mentha spicata* recorded essential oils increase of 64% and 48.85% in the induced polyploid population, respectively (Moetamedipoor et al., 2022; Bharati et al., 2023a). A similar rise in the essential oil of 46% in the induced tetraploid plants was recorded in *Thymus vulgaris* (Homaidan Shmeit et al., 2020). The surge in essential oil production holds the potential to drive enhanced profitability across diverse sectors, including fragrance, cosmetics, aromatherapy, and herbal medicine, which heavily rely on the utilization of lemon balm-derived essential oils.

Determining the nutrient profile in the newly developed genotype of lemon balm holds important significance. Understanding how induced polyploidy affects nutrient composition and uptake provides valuable insights for optimizing cultivation practices to enhance the medicinal quality of the herb. This is particularly pertinent for medicinal plants where the

concentration of bioactive compounds is often linked to nutritional status (Yadegari, 2017; Radha et al., 2021). Furthermore, understanding the nutrient profile of the new genotype with medicinal potential is imperative from a safety perspective. This is because the plant's uptake of both essential and potentially toxic elements is crucial for discerning potential health risks associated with the consumption of these novel medicinal plants of lemon balm (De Souza et al., 2021). In the current study, the tetraploid genotype exhibited a noteworthy increase of 23.57% in potassium content compared with the control diploid genotype. However, no significant changes were observed in other macro and micro-nutrients, which remained unaffected or displayed significant decreases. An increase in the potassium concentration in the induced polyploid population has been previously reported in *Callisia fragrans* (Beranová et al., 2022). In the same study, polyploid genotypes with decreased calcium levels were also observed. It has been previously reported that the polyploidization can influence the metabolic pathways involved in nutrient uptake, transport, and accumulation, which could cause these disparities in the mineral concentration (Tossi et al., 2022).

GC-MS analysis revealed that diploid and tetraploid essential oils had 3 major components: neral, geranial, and citronellal, where neral and geranial increased by 9.49% and 11.06% in the tetraploid plants. Geranial and neral are predominantly present in a well-known monoterpene aldehyde known as citral (3,7-dimethyl-2,6-octadienal) (CTR), mainly found in citrus fruits and herbs. The CTR possesses various beneficial properties, including anti-cancer, antimicrobial, anti-inflammatory, spasmolytic, analgesic, and chemopreventive activities (Bakkali et al., 2008; Liao et al., 2015; Aprotosoaie et al., 2019; Silva et al., 2022). The increased presence of these compounds in the essential oil of tetraploid *M. officinalis* suggests that the newly obtained genotype through polyploidization might potentially display improved therapeutic effectiveness. However, this emphasizes the importance of further systematic research to explore the pharmacological potential of the essential oil produced by tetraploid plants.

6.5 Conclusions

In the present investigation, oryzalin has been identified as a potent anti-mitotic agent within the context of *M. officinalis*. Notably, the treatment with oryzalin successfully induced a tetraploid genotype ($2n = 4\times = 64$) from nodal segments of diploid plants ($2n = 2\times = 32$). The tetraploid genotype exhibited normal growth and maintained a stable ploidy level consistently over time. The tetraploid genotypes displayed significantly thick, large, high number of leaves per plant with significantly larger stomata, higher chlorophyll content and higher photosynthetic

performance. Furthermore, the tetraploid genotypes also exhibited a noteworthy elevation in essential oil content in *M. officinalis*. This synthetic polyploidization process resulted in an increase not only in overall essential oil yield but also in the concentrations of key essential oil components. The obtained genotype in the current study possesses high economic value due to its superior agronomical traits. The obtained genotype can be scaled up for commercial adoption to meet the rising demand and address the limited production, thereby generating significant economic benefits. Chromosome doubling may play a pivotal role in breeding *M. officinalis* and other medicinal and aromatics plants.

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7. Morphological, cytological, and molecular comparison between diploid and induced autotetraploids of *Callisia fragrans* (lindl.) woodson

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This chapter explores the potential of oryzalin mediated in vitro polyploidization in inducing autopolyploid in Callisia fragrans (Lindl.) Woodson and assesses its impact on the morpho physicochemical properties.

Author contribution: Rohit Bharati was involved in conceptualization, data curation, investigation, methodology, software, writing -original draft, writing – review & editing.

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7.1 Abstract

The objective of the current study was to assess the efficiency of oryzalin in inducing polyploids in *Callisia fragrans* (Lindl.) Woodson by *in vitro* polyploidization. Shoot tips were subjected to Murashige and Skoog (MS) medium containing oryzalin at concentrations 1, 5, and 10 µM for 4 and 8 weeks. Further, the ploidy levels of the plants were confirmed using flow cytometry and chromosome counting. Among all treatments, six tetraploid plants (2n = 4x = 24) were obtained after 8 weeks in MS medium containing 5 µM oryzalin. Upon *ex vitro* transfer, tetraploid plants were morphologically distinct compared to diploid plants. The size of the leaf and flower increased significantly and nearly doubled when compared to the mother diploid plant. Further, inductively coupled plasma–optical emission spectrometry showed that tetraploid plants exhibited significantly higher sodium, iron, and calcium content, and the potassium content was increased by 100%. Molecular analysis utilizing iPBS and CDDP markers was tested for the first time in *C. fragrans* to assess the variation between tetraploid and diploid genotypes. Both the markers generated three major clusters, indicating a clear distinction between diploid, tetraploid, and the mixoploid genotypes. In conclusion, *in vitro* polyploidization using oryzalin could effectively induce polyploids in this and related species. Additionally, the results obtained in this study will provide a basis for future breeding opportunities in this species.

Keywords: chromosome doubling; flow cytometry; *in vitro*; oryzalin; polyploidization

7.2 Introduction

Callisia fragrans (Lindl.) Woodson (basket plant; 2n = 2x = 12) is a perennial plant belonging to the Commelinaceae family along with other 500 species. This monocotyledonous plant originated in Mexico and is now widely used across the globe for its medicinal and ornamental properties [1,2,3]. In folk medicine, it is used to treat various conditions such as joint disorders, inflammation, burns, wounds, etc. [3,4]. For instance, leaf extract infused with ethanol is often used to improve immunity and treat joint disorders [4]. Additionally, the ethanolic extract of *C. fragrans* has also recently been reported to possess anti-inflammatory and antioxidant activities [5]. Moreover, a study concluded that *C. fragrans* contains various bioactive components, such as phenolic compounds, amino acids, carbohydrates, flavonoids, coumarins, vitamins, etc. [1]. Studies suggest that these compounds are responsible for the treatment of illnesses such as oncological diseases, cardiovascular problems, tuberculosis, asthma, and gastrointestinal disorders, and the healing of burns and wounds [1,3,6,7].

Medicinal plant breeding focuses on obtaining new genotypes with higher and novel content of secondary metabolites. Several methods are used for plant breeding and genetic improvements such as hybridization, heterogenic breeding, mutation induction, polyploid induction, and genetic engineering. One of the most common methods used for breeding medicinal plants is *in vitro* somatic polyploidization. It is a fast, reliable, efficient, and cheap method of producing novel genotypes with superior agronomical traits. This method is based on the manipulation of somatic chromosomes induced by a wide range of antimitotic agents [8,9,10]. The most commonly utilized antimitotic agent in polyploid induction is colchicine [11,12], although colchicine has various side effects, such as sterility, and toxicity [13]. Hence, recently oryzalin has become an alternative for its effectiveness and fewer side effects [13,14,15]. Polyploidization (chromosome doubling) often causes useful changes such as higher biomass, a higher amount of bioactive compounds, and better environmental (stress) adaptability [9,13,16]. For example, polyploidization increased essential oil content in *Thymus vulgaris* L. and *Chamaemelum nobile* All. [14,17]. Similarly, a higher phenolic acid content (cichoric acid, caffeic acid, chlorogenic acid, caftaric acid, and 1,5-dicaffeoyl quinic acid) in tetraploid *Echinacea purpurea* (L.) Moench [18], total phenolic and flavonoid content in *Salvia officinalis* L. [19], and increased levels of carotenoid content in *Physsalis alkekengi* L. [20] and *Zingiber officinale* Roscoe were observed after chromosome doubling [21].

To date, a few breeding attempts have been made to develop new genotypes in the Commelinaceae family. For instance, studies have utilized gamma irradiations and

colchicine to obtain novel genotypes in *Commelina benghalensis* L. [22,23]. Particularly in *C. fragrans*, few studies have tested colchicine, caffeine, and hydroquinone for their efficacy in producing novel genotypes. However, oryzalin has never been tested for its efficiency in inducing polyploids in this species or plants from this family. Moreover, an *in vitro* approach to polyploidization has also not been tested in the species of study. Hence, the aim of this study was to obtain autopolyploid plants from diploid plants (2n = 2x = 12) via *in vitro* polyploidization using oryzalin as an antimitotic agent and, additionally, to develop an optimized protocol for *in vitro* polyploidization in this and related species. In newly obtained genotypes, various novel morphological, cytological, and molecular variances could be observed.

7.3 Materials and methods

7.3.1 Plant material acquisition, surface sterilization, and culture establishment

Callisia fragrans diploid plants (2n = 2x = 12) with 6 basic number of chromosomes (x = 16) was used [24]. The plants were grown in greenhouse conditions (average temperature 22.5 °C; relative air humidity 70–80%) in plastic containers (5 × 5 cm). After 4 weeks of cultivation, the stolons started to appear. At that development stage, plants were transplanted into larger pots (15 cm in diameter). Over the next eight weeks of cultivation, the stolons grew up to 60 cm in length. After eight weeks, nodal segments were collected and subjected to surface sterilization. Firstly, nodal segments were rinsed with running tap water for 10 min followed by treatment with 70% ethanol (*v/v*) for 2 min. Further, nodal segments were immersed in a solution containing 1% sodium hypochlorite (*v/v*) and two drops of Tween 20 for 10 min. Finally, the nodal segments were rinsed three times using autoclaved double distilled water. A rotatory shaker was used for all the rinses. The sterile nodal segments were then introduced into *in vitro* conditions and then propagated on MS medium [25] supplemented with mg/L 6-benzylaminopurine (BAP) and 0.1 mg/L indole-3-acetic acid (IAA) in Erlenmeyer flasks. Explants were cultivated under controlled conditions (day/night temperature 25 °C/20 °C and day/night photoperiod 16 h/8 h with the light intensity of 57.5 μmoL/s/m^2).

7.3.2 Polyploidy induction

A total of 180 explants (five plants per flask) were exposed to three oryzalin (Sigma-Aldrich, St. Louis, MO, USA) concentrations (1, 5, and 10 μM) and two treatment durations (4 and 8 weeks) on MS media. Oryzalin was added to the freshly prepared media (after autoclaving)

using filter sterilization (0.22 µm). Thirty plants were used for each treatment (concentration × treatment time). Upon the treatment, plants were removed from the oryzalin-containing media and washed with sterile double distilled water under a laminar flow box, and then transferred to oryzalin-free MS media for further propagation. These treated plants were maintained on oryzalin-free MS media for 60 days before ploidy level determination. A control variant where explants were subjected to MS medium without any oryzalin treatment was also used.

7.3.3 Flow cytometry

Fresh leaf samples of approx. 1 cm^2 were used for the ploidy level determination according to the protocol previously reported [**26**]. The sample was placed in a Petri dish and chopped with a sharp razor blade by adding 1 mL of Otto I buffer (0.5% Tween 20 (*v*/*v*), 0.1 M $C_6H_8O_7$) into small fragments, creating a suspension of disrupted leaf tissues. The suspension was then filtered through a nylon filter (50 µm). Further, 1 mL Otto II buffer + 4′,6-diamidino-2-phenylindole solution (DAPI, 2 µg/mL, (*w*/*v*)) was added to the suspension. The prepared solution was then run through a Partec PAS flow cytometer (Partec, Münster, Germany) and a histogram of the relative DNA content was generated using the Flomax software package (Partec, Nürnberg, Germany) (Version 2.3). The control mother plant was used as the control.

7.3.4 Plant transfer into *ex vitro* conditions

Treated and control plants were propagated on MS media. Plants with well-developed root systems were removed from flasks, and washed under running lukewarm tap water to remove residues of media, and transferred into pots (9 × 9 cm) filled with Agro profi RS 1 modified peat substrate (composition: 70% white peat (*w*/*w*); 30% black peat (*w*/*w*); 20 kg/m^3 bentonite; 1.1 kg/m^3 N, P, K (14%, 16%, 18%) (*w*/*w*); 150 g/m^3 Micromax Premium ('s-Gravenzande, Netherlands)) with the addition of Osmocote Exact standard fertilizer (3 kg/m^3, gradual release 8–9 months). The potted plants were covered with plastic foil for one week to ensure sufficient moisture. The plants were then grown under greenhouse conditions (an average temperature of 22.5 °C and natural light). Once a week, the morphological parameters were recorded.

7.3.5 Chromosome counting

Fresh root tips (1 cm approx.) of diploid and tetraploid plants were taken from 7–8 o'clock in the morning and kept submerged in a saturated solution of paradichlorobenzene for 3 h. Thereafter, the root tips were washed 3 times using distilled water and immediately put into a freshly prepared solution of ethanol (96%) and acetic acid (99%) (3:1) for 1 h at room

temperature. The root tips were rinsed again three times using distilled water. For hydrolysis and staining, the tips were incubated in 1N HCl at 60 °C for 15 min followed by washing three times and staining using Schiff reagent for 1 hour. Roots tips were removed from the Schiff reagent and washed again. The tips of the roots were dissected (approx. 0.2 cm) and placed on a glass slide, then a drop of 2% orcein-acetic was dropped onto the root tip and visualized under a BX51 Olympus light microscope (Olympus Optical Co., Tokyo, Japan) at 100× magnification [27].

7.3.6 DNA extraction

Total genomic DNA was extracted from the young leaves of the control, tetraploid, and mixoploid plants. Two plants were used to obtain young leaves for DNA extraction. A mixture of leaf tissue was prepared from 10 randomly chosen discs with 1 cm diameter, obtained from at least 5 leaves. The surface of the discs was disinfected slightly with a 20% SAVO solution (commercial bleach) (1% NaClO) (*v/v*). DNA was extracted by a GeneJET plant DNA purification kit (ThermoScientific, Waltham, MA, USA) according to the instructions of the manufacturer. The quantity and quality of extracted DNA were measured by a NanoDrop P500 (Implen GmbH, München, Germany).

7.3.7 iPBS analysis

Six different primers previously reported for inter-primer binding site polymorphism analysis (iPBS) were firstly tested for *C. fragrans* fingerprints [28]. PCRs were prepared by EliZyme Robust HS (Elizabeth Pharmacon, Brno, Czech Republic) together with 800 nM of iPBS primer and 50 ng of DNA in each reaction. PCR conditions of the iPBS were as follows: 95 °C for 5 min; 45 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min; and 72 °C for 10 min. The PCRs were repeated twice.

7.3.8 CDDP analysis

The conserved DNA-derived polymorphism (CDDP) method was used to analyze the variability of *C. fragrans* control plants with the polyploid genotypes generated utilizing five previously reported primer combinations (WRKY-F + WRKY-R1, WRKY-F + WRKY-R2a, WRKY-F + WRKY-R2b, WRKY-F + WRKY-R3, WRKY-F + WRKY-R3b) [29]. PCRs were prepared by EliZyme Robust HS (Elizabeth Pharmacon) together with 400 nM of each primer and 50 ng of DNA in each reaction. Time and temperature profiling of the PCRs was as follow:

95 °C for 5 min; 40 cycles of 95 °C for 45 s, 54 °C for 45 s, 72 °C for 90 s; and 72 °C for 10 min. The PCRs were repeated twice.

7.3.9 DNA fingerprinting

The obtained iPBS and CDDP fingerprint data from 2% agarose electrophoresis (*w/v*) were analyzed, and the generated profiles were converted into 0–1 binary matrices. The unweighted pair group method with arithmetic mean (UPGMA) analysis was performed, and the Jaccard coefficient of genetic similarity (1945) was used for dendrogram construction. The dendrograms were prepared in the free DendroUPGMA software (http://genomes.urv.cat/UPGMA/ (accessed on 20 July 2022)).

7.3.10 Mineralization and ICP-OES elemental analysis of micro- and macronutrients

Approximately 0.25 g of each sample was weighed on an ABT-120/5DW analytical balance (Kern & Sohn, Balingen, Germany) and transferred to polytetrafluoroethylene (PTFE) mineralization tubes. Mineralization was performed with pressure microwave digestion on EthosOne (Milestone, Sorisole, Italy) in 5 mL concentrated nitric acid 69% (*v/v*) and 1 mL of 30% hydrogen peroxide with 2 mL of ddH_2O. The obtained digestate was filtered through Filtrak 390 filter paper. Elemental analysis was performed on an Agilent 720 ICP-OES spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA) with axial plasma configuration and with an SPS-3 autosampler (Agilent Technologies Inc., CA, USA). Three replications of each genotype were carried out.

7.3.11 Statistical analysis

Statistical analysis of the data on growth parameters obtained was performed using the statistical software Statistica® (version 13.6). The Kruskal–Wallis test was used to compare the differences between treatments. For the test, differences were considered as significant at $p < 0.05$.

7.4 Results

7.4.1 Chromosome doubling

A total of 180 plants were exposed to oryzalin at three concentrations and two treatment durations. The survival rate was 100% in all treatments tested. During cultivation on media with

oryzalin, the plants generated offshoots (on average 1.7 per plant). In total, 306 plants were regenerated from all treatments. All treated plants were tested by flow cytometry (Figure 7.1).

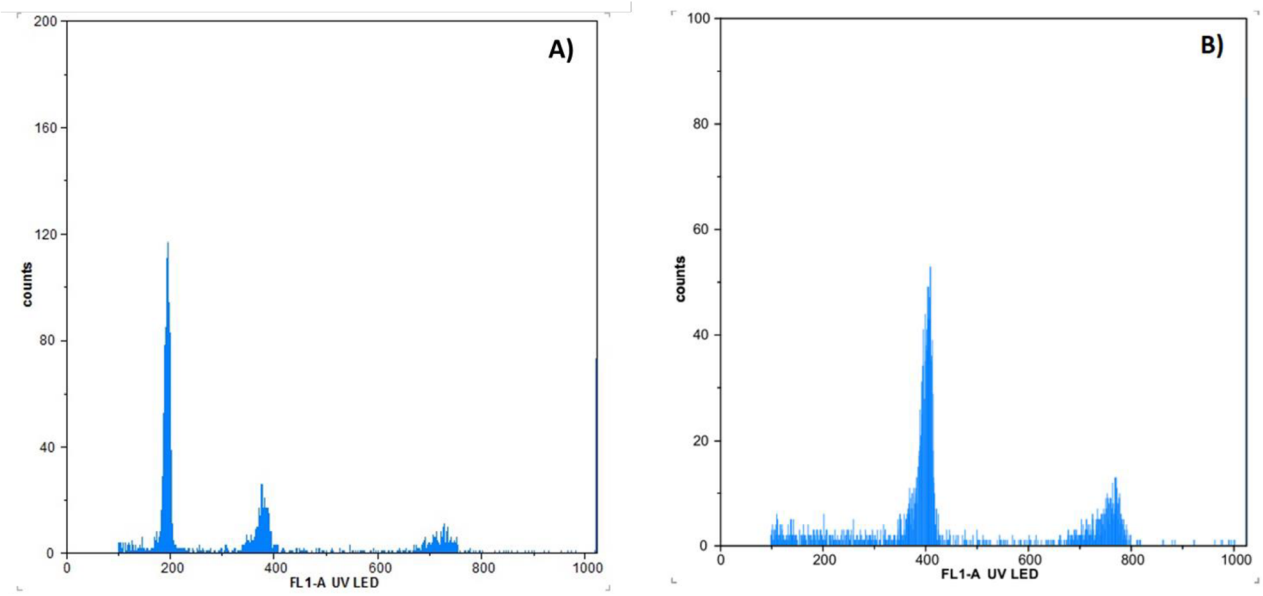

Figure 7.1 Histogram of flow cytometry analysis from *C. fragrans*: (A) histogram of control plant (diploid) and (B) polyploid plants (tetraploid).

In total, six autotetraploid and two mixoploid plants were obtained from diploid mother plants across all the treatments. Samples with multiple peaks were considered as mixoploids. All autotetraploid genotypes were obtained from treatment of 5 µM oryzalin for 8 weeks, and mixoploid plants were obtained from treatment of oryzalin at 5 µM for 4 weeks and 10 µM for 8 weeks. To validate the results from flow cytometry, direct chromosome counting was performed. It was confirmed that the diploid plantlets were 2n = 2x = 12, and after successful polyploid induction, autotetraploid plantlets had 2n = 4x = 24 chromosomes in the somatic cells (Figure 7.2A,B). Results are summarized in Table 7.1. The newly obtained six tetraploid genotypes were marked as P1–P6 and two mixoploids as M1–M2. Genotype P6 was not viable, exhibited slower growth, and was unable to create offshoots; hence, it was excluded from the study. All genotypes were transferred into *ex vitro* conditions with a 100% survival rate.

Table 7.1 Effect of oryzalin treatment on polyploid induction of ***Callisia fragrans***.

Concentration (µM)	**Duration (weeks)**	**No. of plants**	**No. of plants after treatment**	**Survival rate (%)**	**Mixoploid plants**	**Tetraploid plants**	**Efficiency rate (%)**
1	4	30	34	100	0	0	0

	8	30	66	100	0	0	0
5	4	30	47	100	1	0	2.13
	8	30	63	100	0	6	9.52
10	4	30	38	100	0	0	0
	8	30	58	100	1	0	1.72
Total		180	306	100	2	6	

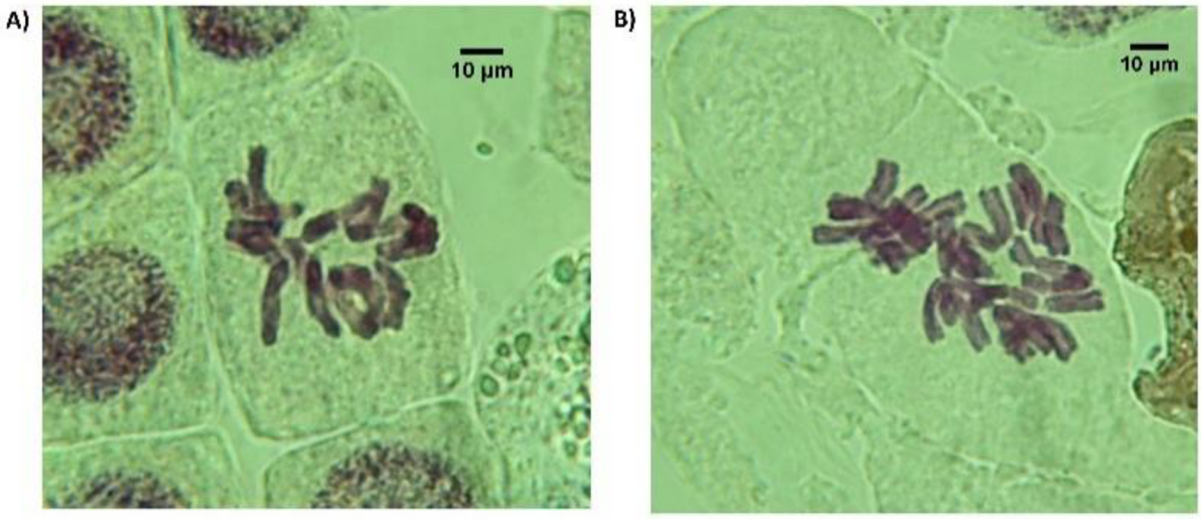

Figure 7.2 Chromosomes of *C. fragrans* of (A) control (diploid) and (B) polyploid plants (tetraploid) under 100x magnification.

7.4.2 Morphological differences between diploid and the autotetraploid genotypes

Polyploid plants exhibited notable morphological differences in comparison to the control genotype. The evaluation of growth variability is summarized in Table 7.2 and Table 7.3. Significantly reduced numbers of leaves were detected in P3, P4, M1, and M2 genotypes when compared to the diploid genotype. However, the average leaf length of all polyploid genotypes (P1–P4, M1, M2) increased significantly (5 cm on average), except genotype P5 which exhibited significantly shorter leaves (22.35 ± 0.94 cm) among all the genotypes. With respect to the height of the plants, the autotetraploid plants did not exhibit any significant difference from the diploid plants although the mixoploids grew shorter and were characterized by more compact growth compared to control plants (46 ± 7.76 and 46 ± 7.75 cm). Similarly, polyploid genotypes did not differ in the average number of internodes except for P4 and mixoploids (M1 and M2) for which it was less than that of diploid plants (Table 7.2, Figure 7.3). All the induced

genotypes produced shorter stolons compared to the control genotype. The longest and shortest stolons were observed in the control (76.1 ± 10.04) and P5 genotypes, respectively (Table 7.2). However, the average number of stolons per plant remained unchanged across all the genotypes as no statistically significant differences were observed.

The polyploid plants were characterized by a larger flower size. For example, tetraploid P3, when compared with diploids, showed a significant increase in diameter (66.88%). The P3 genotype grew longer and wider flower petals (Figure 7.4, Table 7.3). However, the longest flower petals were measured in the P1 genotype (6.045 ± 1.01 mm). Genotypes P2, M1, and M2 did not bloom at all. Interestingly, the control genotype, P1, and P3 started flowering two weeks earlier than P4 and P5.

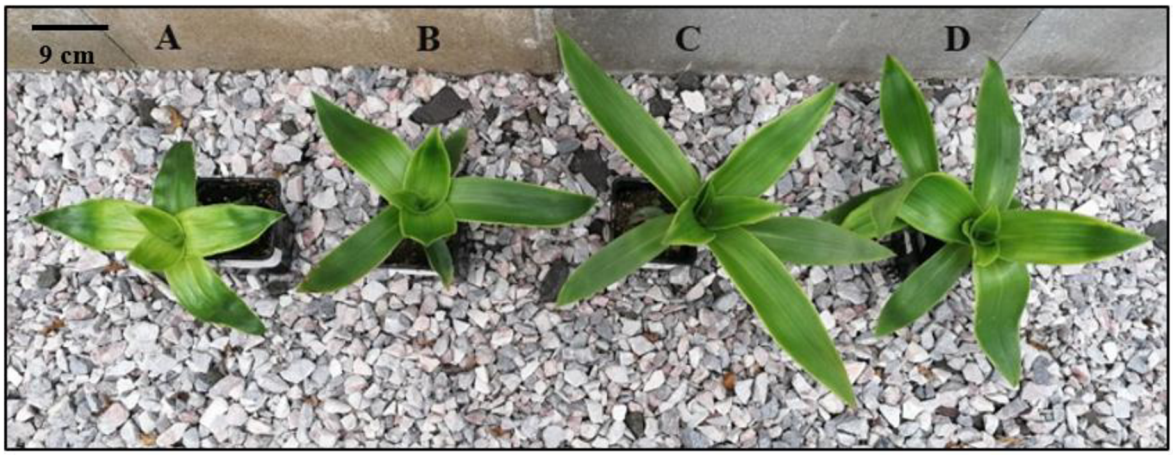

Figure 7.3 Two weeks old *C. fragrans* plants in *ex vitro* conditions: A) control plant; B) tetraploid genotype P2; C) tetraploid genotype P3; D) mixoploid genotype M1.

Table 7.2 Morphological characteristics of diploid, mixoploid and autotetraploid plants cultivated in a greenhouse.

Genotype	Average number of internodes	Average number of stolons	Average number of leaves	Average height (cm)	Average length of stolons (cm)	Average number of internodes on stolon	Average leaf length (cm)
Control	16.3 ± 1.7 a	5.5 ± 1.35 a	23.7 ± 2.26 a	64 ± 6.58 a	76.1 ± 10.04 a	10.33 ± 0.92 a	24.47 ± 1.48 c
P1	14.8 ± 2.15 a	4.4 ± 1.17 a	22 ± 1.76 a	67.5 ± 10.61 a	54.95 ± 10.8 b	9.51 ± 0.86 a	30.87 ± 0.42 a
P2	14.3 ± 1.16 a	4.3 ± 1.25 a	20.9 ± 2.08 a	62.8 ± 6.09 a	57.012 ±16.87 b	6.92 ± 2.3 c	28.69 ± 0.88 b
P3	14.7 ± 0.68 a	5.6 ± 0.52 a	17.9 ± 0.87 b	61.9 ± 3.,48 a	64.133 ±5.64 b	9.33 ± 0.54 a	31.86 ± 1.57 a
P4	11.9 ± 1.37 b	4.5 ± 0.53 a	17 ± 0.94 b	62 ± 10.33 a	61.48 ± 9.92 b	8.19 ± 0.94 b	31.34 ± 0.82 a
P5	17.3 ± 1.34 a	4.5 ± 0.85 a	21.3 ± 1.49 a	64.5 ± 5.93 a	48.835 ± 5.69 c	8.3 ± 2.76 a	22.35 ± 0.94 c
M1	12.3 ± 1.9 b	5.2 ± 0.42 a	18.8 ± 3.36 b	46 ± 7.76 b	56.86 ± 15.48 b	8.62 ± 1.35 a	32.7 ± 1.14 a
M2	12.2 ± 1.81 b	6 ± 1.25 a	18.2 ± 2.49 b	46 ± 7.75 b	57.38 ± 13.73 b	8.82 ± 1.21 a	32.73 ± 1.61 a

*Different superscript letters within the same column differ significantly (Kruskal-Wallis test, $p < 0.05$).

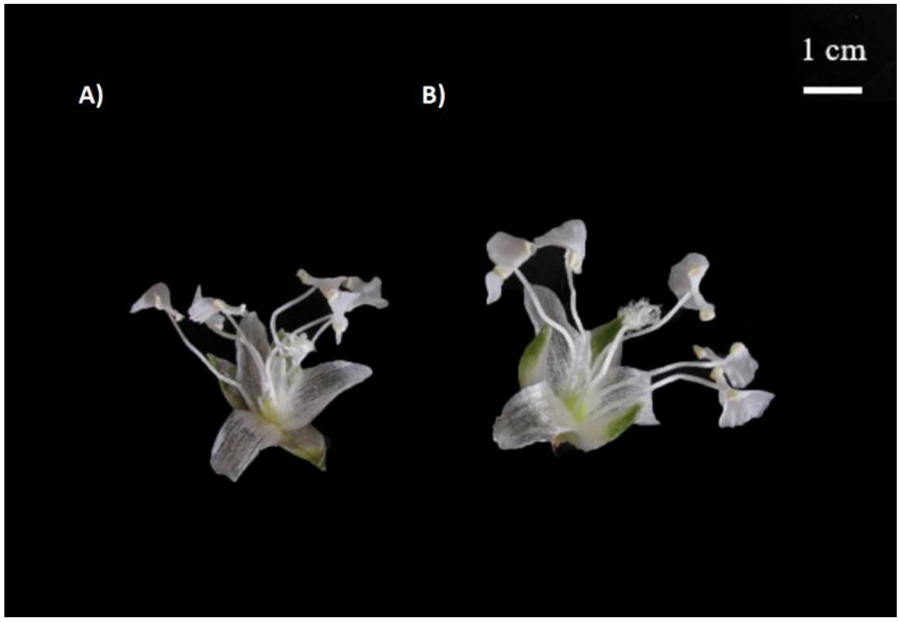

Figure 7.4 Morphological variation between diploid (A) and induced tetraploid (B) flowers of *C. fragrans* cultivated in greenhouse conditions.

Table 7.3 Morphological characteristics of flowers of diploid and autotetraploid plants.

Genotype	Average diameter (cm)	Petal length (mm)	Petal width (mm)
K	2.08 ± 0.18b	3.54 ± 0.41b	1.94 ± 0.37b
P1	2.08 ± 0.16c	6.045 ± 1.01a	2.33 ± 0.88b
P3	3.11 ± 0.10a	5.96 ± 0.58a	2.68 ± 0.55a
P4	2.22 ± 0.16b	5.97 ± 0.99a	2.42 ± 0.54b
P5	1.92 ± 0.09c	4.94 ± 0.55b	2.41 ± 0.77b

*Different superscript letters within the same column differ significantly (Kruskal-Wallis test, $p < 0.05$).

7.4.3 iPBS analysis (Inter-Primer Binding Site Polymorphism)

Six previously published iPBS primers were used for the analysis (primers: 1838, 1846, 1882, 2270, 1897, and Frodo2) [**28**]. Primer 1882 generated a monomorphic profile for all of the analyzed polyploid plants of *C. fragrans* accessions (P1–P4, M1–M2) with two (80 bp plus 90 bp) amplicon deletions in the control plant. In the control plant, nine fragments were amplified, and in the polyploid accessions, 11 iPBS fragments were amplified. A completely monomorphic iPBS profile was obtained for the primer 1897 with seven generated amplicons across all the

genotypes. Primer Frodo2 generated a different amplified iPBS profile for the accession M2, where deletions of short fragments occurred, but all of the other samples have 12 iPBS fragments per accession. Three of the remaining iPBS primers yielded polymorphic profiles among the analyzed *C. fragrans* plants. Primer 1838 generated 88% polymorphism and amplicons varied from three (control) up to eight (P4) per accession. Insertion of the 260 bp fragment was achieved for the M2 and P4 plants and a 500 bp insertion was obtained for the plants P1–P4. The length of the generated amplicons varied from 45 bp up to 990 bp. Primer 2270 was characterized by 55% polymorphism but provided a different iPBS profile for the control *C. fragrans* plant, where two deletions occurred. Profiles of M1–M2 and P1–P5 were monomorphic. The number of generated amplicons was five for control plants and seven for polyploid plants. Primer 1846 provided 87.5% polymorphism and was the most variable among the used iPBS primers. No specific pattern was found between the polyploid genotypes and the control plant of *C. fragrans*. Control plants were not especially different in their iPBS profile, but here, the most amplicons were obtained (11) and the rest of the samples provided from four up to nine amplicons in iPBS. Genotypes M1, P2, and P4 have similar, but not the same, profiles and plants P1 and P5 generated the same iPBS profiles. A dendrogram (Figure 7.5) was constructed for the analyzed plant variants based on the results of iPBS fingerprinting. Three main branches were generated where the control plant was clearly separated from the treated polyploid plants. Further, two branches separated the tetraploids (P1–P5) from the mixoploids (M1–M2). The P1–P5 plants were divided into two smaller subclusters with the same profile for P1 and P2.

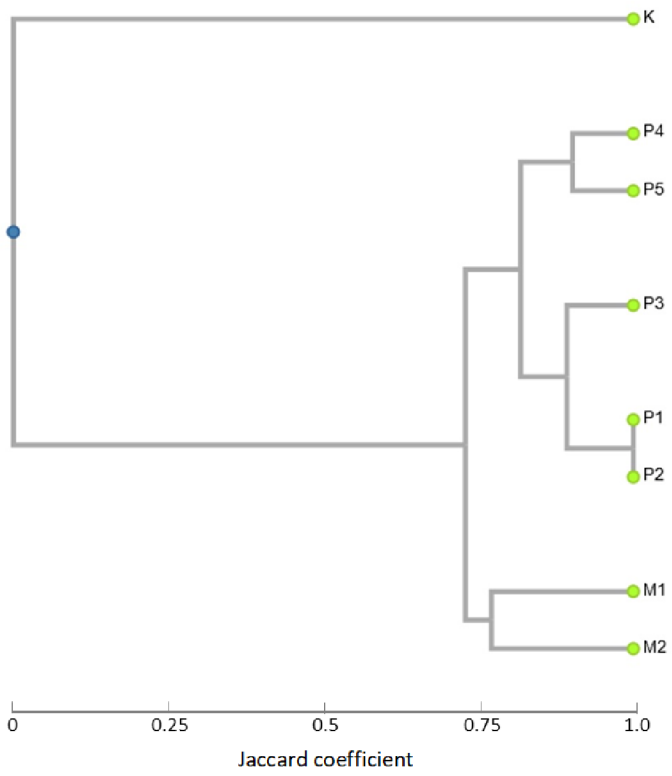

Figure 7.5 Dendrogram of analyzed *C. fragrans* genotypes based on iPBS markers.

7.4.4 Conserved DNA-derived polymorphism (CDDP) analysis

In this analysis, five standard primer combinations were used to generate CDDP fingerprint profiles of the coding regions of the *C. fragrans* genome. In four of them, the control *Callisia* plant was differentiated by this technique by a specific locus amplification pattern.

Primer combination WRKY-F + WRKY-R1 provided a 430 bp insertion in the profile of the control plant, but the other amplicons correspond fully with the profile of M1 and M2 plants. In the case of P1–P5 genotypes, variability was obtained in the short fragments, deletion of a 490 bp locus was generated, and a 510 bp locus has stronger amplification when compared with the other analyzed *C. fragrans* accessions. Primer combination WRKY-F + WRKY-R2a resulted in the monomorphic profiles of M and P plants and the generated CDDP profile of the control plant differs in the insertion of one locus of 490 bp and two loci of approximately 130 bp. Primer combination WRKY-F + WRKY-R2b provided differentiated fingerprints for the control plant and P1. The control plant was typical by insertion of a 240 bp locus and deletion of a 260 bp locus. Mixoploid plants and P2–P5 plants have monomorphic profiles and the P1 plant is different from them by the shift of the CDDP fingerprints and insertion of 130 bp and

140 bp loci. Primer combination WRKY-F + WRKY-R3 generated monomorphic profiles for both M and P plants, too. The control plant is different from them by insertion of 150 bp and 370 bp loci. Primer combination WRKY-F + WRKY-R3b provided a completely monomorphic profile of all analyzed *C. fragrans* plants (new genotypes and control plants).

A dendrogram (Figure 7.6) was constructed for the analyzed *C. fragrans* plants based on the results of CDDP fingerprinting. Three main branches were generated with the CDDP profile from the most distinct control plant in comparison with the rest of the analyzed plants. The P1–P5 genotypes were divided into two smaller subclusters with the same profile for P2, P3, and P5. Mixoploid genotypes have the same profiles. Both of used marker techniques generated fingerprints that were able to distinguish different polyploidy backgrounds of analyzed accessions. The individual characteristics of both are summarized in Table 7.4.

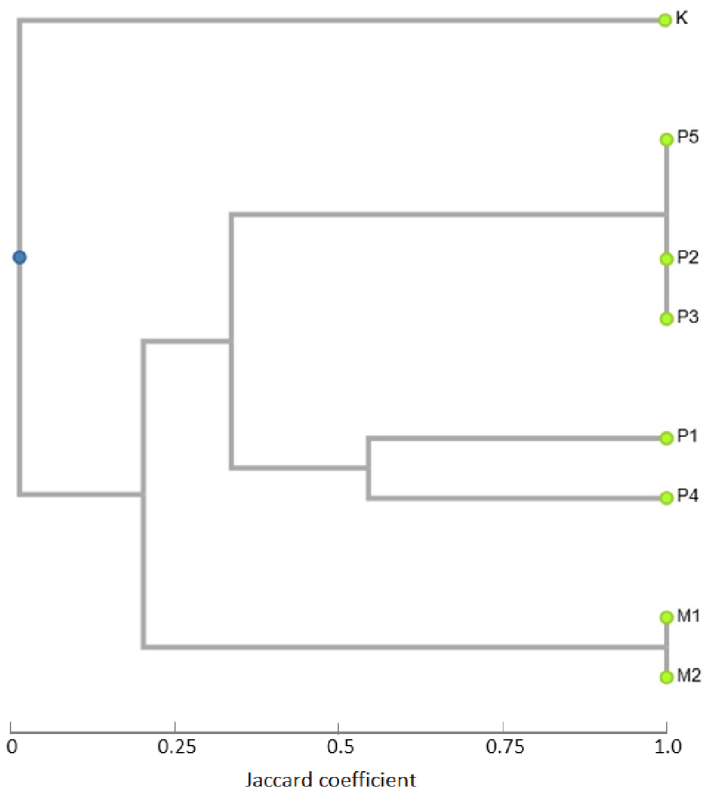

Figure 7.6 Dendrogram of analyzed *C. fragrans* genotypes based on CDDP results.

Table 7.4 Characteristics of fingerprints obtained by iPBS and CDDP markers.

Technique	Primer/Primer combination	Polymorphism (%)	Min length of generated fragments	Max length of generated fragments	Average Dice coefficient of genetic similarity
iPBS	1846	100	69	1190	0.52
	2270	100	68	789	0.61
	1882	98	60	820	0.95
	1854	99	64	988	0.65
	1867	99	71	1850	0.89
	1868	100	90	1230	0.88
CDDP	WRKY-F+R1	96	55	560	0.52
	WRKY-F+R2a	95	82	780	0.45
	WRKY-F+R2b	99	70	930	0.36
	WRKY-F+R3	99	50	520	0.49
	WRKY-F+R3b	93	63	900	0.58

7.4.5 Nutrient content

The nutrient content analysis detected variance in the content of macro- and micronutrients among the studied genotypes. Potassium content showed an almost 100% increase in all polyploid genotypes. The calcium content increased only in the P1 genotype (10.97%) compared to the diploid. Other polyploid and mixoploid genotypes had calcium content reduced in comparison with diploid plants (Table 7.5). The average sodium content in all the induced genotypes was observed to increase approximately four to six times compared to the control genotype. The summary of nutrient content is given in Table 7.5.

Table 7.5 Macronutrient, micronutrient, and fragment compounds analyzed from leaves of diploid, tetraploid, and mixoploid *C. fragrans* plants (mg.kg−1).

Nutrient	Control (K)	P1	P2	P3	P4	P5	M1	M2
K	15772.00±174.00g	31040.35±92.75d	37369.05±68.15b	30066.50±39.50e	39041.80±9.40a	39047.45±15.05a	33313.35±5.95c	26340.25±10.05f
Ca	23253.40±105.50b	25777.90±89.40a	8862.80±4.70g	13685.25±75.05e	13122.80±10.30f	17231.55±5.65c	17334.80±11.60c	14798.30±10.90d
Mg	12866.10±120.50b	14295.50±15.00a	4868.00±10.00h	8039.85±13.35f	6882.75±5.55g	10316.95±6.25c	9353.75±3.05d	8517.55±4.15e
Na	195.95±1.55h	1368.15±2.95de	1758.25±12.85a	1305.35±7.85f	1706.50±3.80b	1404.50±4.70cd	1351.85±2.55e	922.20±1.50g
Fe	82.80±0.88d	73.88±0.41e	90.95±0.15c	57.97±0.00f	73.97±0.37e	82.03±0.05d	92.98±0.35bc	93.74±0.00ab
Mn	102.40±0.70e	107.15±0.25e	142.90±0.30b	90.25±0.55f	118.90±0.30d	79.05±0.25g	188.75±2.55a	135.22±0.22c
Zn	48.96±0.45c	43.20±0.25d	53.42±0.46b	36.97±0.10f	56.13±0.20a	44.01±0.15d	50.91±0.25c	39.86±0.41e
Cu	26.76±0.22d	34.73±0.35c	54.16±0.00a	26.05±0.25d	33.12±0.77c	25.07±0.08d	43.32±0.14b	41.93±0.10b
Cr	0.15±0.00f	0.17±0.00cdef	0.16±0.00ef	0.12±0.01g	0.18±0.00bcde	0.08±0.00h	0.17±0.01def	0.21±0.01a
Mo	0.40±0.01f	0.52±0.01de	0.46±0.01ef	1.02±0.01b	0.15±0.00g	ND	0.70±0.02c	1.39±0.02a
Co	ND	0.29±0.00b	ND	0.20±0.00c	ND	0.12±0.01d	0.35±0.01a	ND
Ag	ND	0.07±0.00a	ND	ND	ND	0.07±0.00a	0.11±0.00a	0.02±0.00a
Sr	49.07±0.10d	43.65±0.40f	54.03±0.15b	38.05±0.10g	56.07±0.14a	46.08±0.14ce	50.95±0.29c	38.56±0.40g
Sb	ND	1.98±0.05a	1.90±0.01a	1.72±0.01b	1.44±0.01c	0.91±0.01d	0.47±0.00e	ND
Li	12.04±0.15a	8.07±0.05c	2.44±0.03g	7.68±0.04c	4.04±0.01f	4.85±0.02e	6.22±0.09d	9.76±0.08b
Ba	8.77±0.35a	7.25±0.10b	3.10±0.03d	5.31±0.02c	4.97±0.01c	5.28±0.04c	5.36±0.06c	4.75±0.02c
Al	36.12±0.20a	11.20±0.23e	17.14±0.21c	16.23±0.04c	13.93±0.05d	13.98±0.15d	19.05±0.07b	13.63±0.11d
Others	As 2.21 Pb 0.44	As 1.23 Cd 0.11 Ni 0.45	As 1.76 Ni 0.26 Pb 0.74	Pb 0.67	ND	ND	Cd 0.06 Ni 0.23	Pb 0.28

ND-Not detected and were considered zero for statistical analysis; Nutrients in row "others" were not included in the statistical analysis. *Different superscript letters within the same row differ significantly (Kruskal-Wallis test, $p < 0.05$).

7.5 Discussion

There is no information about *in vitro* induced somatic polyploidy in *C. fragrans* nor in other species from the *Callisia* genus. The experiment of *in vitro* induced somatic polyploidy in *C. fragrans* using oryzalin was carried out for the first time in the current study. Recent articles report that *in vitro* induced somatic polyploidy is often used for breeding medicinal plants from other genera and families such as *Allium cepa* L. [30], *Moringa oleifera* L. [31], *Chamaemelum nobile,* [17], and *Echinacea purpurea* [18]. For this purpose, oryzalin and colchicine are effectively used as antimitotic agents for obtaining new genotypes. Nevertheless, in medicinal plants, it is reported that oryzalin has become a standard preference over colchicine due to its lower toxicity [10,32]. Moreover, it has been extensively used in the breeding of numerous medicinal plants, for example, *Calendula officinalis* [11] and *Cnidium officinale* [33]. Additionally, oryzalin also worked effectively for the chromosome doubling of *Scutellaria barbata* D. [34], *T. vulgaris* [14], and *Rubus sanctus* Schreb. [35]. Interestingly, a study reported that by using oryzalin for polyploidization, the efficiency of obtaining polyploids was raised by 5% in *Spathiphyllum wallisii* Regel [36]. Similarly, oryzalin has also been found to be more effective than colchicine in genome doubling *of Smallanthus sonchifolius* (Poepp.) H. [37], *Solanum* species [38], and *Agastache foeniculum* (Pursh) Kuntze [12].

From the results of the current study, oryzalin added into media appears as an effective antimitotic agent for polyploidization of *C. fragrans*. The addition of 5 µM of oryzalin directly into the media for 8 weeks of treatment time was found to be the most efficient treatment for inducing polyploidy in *C. fragrans*. Despite the fact that the higher concentration was not toxic for the plants, there were no obtained tetraploid genotypes from the higher concentrations. Similar to the current study, the addition of oryzalin in media was successful for polyploidization of *Anemone sylvestris* L. [26] and, *S. wallisii* [36]. Polyploidization in *Anemone sylvestris* was significantly higher at medium concentrations of 5 µM and 10 µM compared to the higher concentrations studied. The highest concentration (15 µM) for the 12 weeks' duration was toxic for all treated plants. The shorter time was sufficient for the induction of tetraploid plants (8 weeks) with increasing concentrations of oryzalin [26]. The lower concentrations were also successfully used for inducing polyploidy in *M. oleifera* where tetraploid plants were obtained in the treatment of seeds for 1 day with 15 µM and 60 µM of oryzalin [31].

Callisia fragrans plants were well adapted to *ex vitro* conditions. The survival rate of transferred plants was 100% for all the variants. As previously reported, the autotetraploid plants of *Anemone sylvestris* grown in field conditions had a 100% survival rate in the next year, while the diploids had a survival rate of only 58% [26].

The newly obtained genotypes in the current study have significant morphological differences. The number of leaves decreased in tetraploid plants of *C. fragrans*. Although a previous study reported that despite the number of leaves of tetraploid plants of *Centella asiatica* L. decreasing, the fresh weight of tetraploid plants increased by more than 77% [39]. A similar increase in biomass by polyploidization was also reported in *Salvia miltiorrhiza* and *Scutellaria baicalensis* Georgi [40,41]. All genotypes of induced autotetraploid plants of *C. fragrans* exhibited an increase in leaf length except P5. Similar results were obtained in *Thymus vulgaris* [14], *Salvia miltiorrhiza* [40], and *Raphidopus persicus* [35]. Induced tetraploid genotypes of *Moringa oleifera* also generated larger leaflets [31]. Similarly, autotetraploid genotypes of *C. fragrans* also generated significantly larger inflorescences with larger petals; only one genotype (P5) generated flowers smaller than the control plant. Previously, a significant increase in flower size was also observed in *A. sylvestris* [26] and *C. nobile* [17]. By polyploidization of *Rubus* spp., the crown diameter increased by 100% in *Rubus caesius* L. and, on the contrary, *Rubus hirtus* Rchb. exhibited a decrease in flower size [35].

A variance in the content of macro- and micronutrients was detected. Potassium showed an almost 100% increase in all polyploid and mixoploid genotypes. The calcium content increased only in the P2 genotype. The other genotypes had their calcium content reduced in comparison with diploid plants. Similarly, a previous study reported that upon successful polyploid induction in *Moringa oleifera*, protein content increased by 20%, fat content increased up to 34%, and calcium content increased up to 20% [31]. Additionally, the amount of chemical compounds has been reported to increase in several medicinal plants such as *T. vulgaris* [14] and *S. miltiorrhiza* [40]. Similarly, content of matrine and oxymatrine increased by polyploidy induction of the Chinese medicine plant *Sophora tonkinensis* Gagnep., where the oxymatrine content was increased by 107.1% in comparison with the control plant [42]. The biochemical compounds of tetraploid *C. fragrans* and their comparison with the diploid genotype need to be a part of further research.

In both of the DNA-based fingerprints methods used, control plants were separated from polyploid ones. Polyploidization in its natural or *in vitro* conditions results in genome duplication and is an inevitable part of plant evolution and speciation. Different marker

techniques have been used to analyze artificially produced polyploid plants. For instance, microsatellite-based markers were used to characterize the fingerprint profiles of polyploid and aneuploid seedlings from seven diploid *Malus* populations [43] and *Aronia melanocarpa* genetic structure, among the native polyploidy populations, was analyzed by randomly amplified polymorphic DNA (RAPD) [44].

Currently, there are no specific DNA markers reported for *C. fragrans*, therefore nonspecific markers can be utilized for the analysis of its genome variability. By iPBS markers, the variability of the retrotransposon insertion patterns was analyzed in the diploid and tetraploid plants of *C. fragrans*. Here, iPBS analysis was applied for the first time to test the stability of induced polyploid plants of *C. fragrans*. A completely monomorphic iPBS profile was obtained for the primer 1897 which generated seven amplicons. The iPBS fingerprinting showed variability between all genotypes and the control plant, thus the control plant was evidently different from all other genotypes. However, P1 and P2 showed the same profile by iPBS markers. To date, many plant species have been analyzed by iPBS markers, such as *Liparis loeselii* L. [45], *Saussurea esthonica* Baer ex Rupr. [46], and *Prunus armeniaca* [47].

Similar to the iPBS, CDDP was applied for the first time to analyze the *C. fragrans* genome. The used primer combinations did not yield any complete monomorphic profile across the genotype, although some monomorphic bands were obtained for polyploid plants. Three main branches were generated with the CDDP profile, and the control plant-generated fingerprint was clearly separated.

Both of the used marker techniques were effective in distinguishing the control and the polyploid plants and there were only a few differences among them in generated polymorphism as well as in the amplified length of loci. The most important difference was in the similarity coefficients, where the iPBS technique provided a wider individual range of this coefficient and CDDP was narrower in the generated fingerprints.

Different marker techniques have been used to analyze the polymorphism generated through the coding parts of the plant genomes, such as SCoT [48], CDDP [49], TRAP [50], or PBA [51]. Additionally, there are lots of methods using DNA markers for the detection of genetic changes such as inter-simple sequence repeat (ISSR) [52], simple sequence repeat (SSR) [53], randomly amplified polymorphic DNA (RAPD) [54], and amplified fragment length polymorphism (AFLP) [55]. All of them were reported to be reliable [28,56,57], and could be alternatives for

analysis of genotype stability of *C. fragrans* in future studies besides the iPBS and CDDP markers used in this study.

7.6 Conclusions

This is the first report of the successful *in vitro* induction of autotetraploids (2n = 4x = 24) in *Callisia fragrans* (Lindl.) Woodson using oryzalin. Oryzalin was found to be effective in inducing polyploidy in this species under *in vitro* conditions. The newly obtained genotypes are morphologically different from the diploid genotype. Additionally, the polyploids exhibited a different nutrient content profile than the diploid genotype. DNA-based analysis of polymorphism was performed by iPBS and CDDP markers, which were applied for the first time for *C. fragrans*. Hence, the *in vitro* oryzalin-induced polyploidization could be a valuable breeding strategy for *C. fragrans* to produce improved clones with new features. Additionally, the developed protocol might be effective for polyploid induction in plants from the same or a similar family.

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8. Artificial polyploidization enhances morphological, physiological, and biological characteristics in *Melothria scabra* Naudin

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This chapter explores the potential of oryzalin mediated in vitro polyploidization in inducing autopolyploid in Melothria scabra Naudin and assesses its impact on the morpho physicochemical properties with special emphasis on the fruits.

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Abstract

Cucamelon (*Melothria scabra* Naudin, Cucurbitaceae) is a climbing, herbaceous perennial plant with various culinary and medicinal uses. The current study aimed to develop novel autopolyploid genotypes of *M. scabra* through *in vitro* polyploidization and assess their morphological and phytochemical characteristics. For polyploid induction, oryzalin was employed as an antimitotic agent, and it was applied at various concentrations (40, 60, and 80 µM) and durations (24 and 48 h). Flow cytometry analysis confirmed the successful induction of polyploids, with polyploidization efficiency ranging from 2.5% to 15%. From a total of 240 treated plants, a total of 20 autotetraploid plants were obtained. The obtained polyploid and control diploid genotypes were cultivated under greenhouse conditions. Further, the plants were transferred to field conditions, and the leaves, flowers, and fruits were harvested to be evaluated for the morphological, biochemical, and biological activity variations among the obtained genotypes. Morphological comparisons between diploid and autotetraploid plants revealed significant differences in flower characteristics, fruit attributes, and leaf morphology. Nutritional evaluation demonstrated enhancement of key parameters in the induced polyploids compared to the diploid plants, including glucose, fructose, carotenoid, polyphenol, and antioxidant contents, highlighting the potential impact of polyploidization on these traits. The results from this study highlight the potential of artificial chromosome doubling as an effective breeding strategy in *M. scabra* and related plant species.

Keywords: chromosome doubling; cucamelon; oryzalin; polyploidization; plant breeding

8.1 Introduction

Cucamelon, also known as Mexican miniature watermelon (*Melothria scabra* Naudin), is a horticultural crop from the Cucurbitaceae family [1]. It is a diploid (2n = 2× = 24) plant species that originated from Mexico and Central America but has spread widely across other regions of the world [2]. Cucamelon is cultivated for its diminutive, edible fruit, which resembles a miniature striped watermelon (*Citrullus lanatus*). Morphologically, the fruit is oval with an average length of 2.4 cm and a width of 1.5 cm and is consumed either in its raw form or processed as pickles and vegetables [1,3]. The fruits of *M. scabra* contain alkaloids, tannins, flavonoids, terpenoids, and saponins [1]. Apart from these phytochemicals, cucamelons possess numerous ethnopharmacological properties. For example, the leaves of these plants have antidiabetic activity [4], it is used to reduce blood pressure in Sulawesi medicinal folklore, and the extract of cucamelon plant is also applied as a mosquito repellent in some parts of the world [1].

Although cucamelons have numerous uses, they have not benefited from systematic breeding attempts, unlike the major crops like wheat or rice. This neglect presents a compelling opportunity to explore the untapped potential of this crop. In this scenario, artificial chromosome doubling using antimitotic agents offers an exciting avenue. Artificial chromosome doubling or polyploidization is a fast and cost-effective approach in plant breeding for generating novel genotypes with superior traits in relatively less time [5–8]. Artificial polyploidy induction has been a great breeding strategy, particularly in horticultural crops [9], where it has successfully yielded genotypes with novel morphological, physiological, and phytochemical characteristics [10–13].

The most common antimitotic agent used to induce autopolyploid plants is colchicine due to its thermostability and effectivity [12,14]. However, due to its toxic nature and higher binding affinity to animal cell tubulin protein than to plant tubulin, other antimitotic agents like oryzalin are preferred [13,15]. In recent years, the application of oryzalin has gained popularity for chromosome duplication due to its higher success compared to the predominantly used colchicine, particularly within the Cucurbitaceae family [16–19]. For example, a previous study reported a comparably high tetraploid induction efficiency (up to 14%) in *Cucumis melo* var. Makuwa, leveraging the application of oryzalin in conjunction with amiprophos-methyl (APM), when compared to traditional colchicine treatment [20]. Similarly, another study noted a higher effectiveness of chromosome doubling in *Cucumis sativus* L. through the utilization of oryzalin in contrast to trifluralin and colchicine in their study [16]. These collective findings

underscore the promising potential of oryzalin as a valuable alternative for efficient polyploid induction and its relevance to crop improvement and breeding programs.

Artificial polyploidization has never been carried out in *M. scabra* to date. However, several attempts towards polyploid induction in plants from the Cucurbitaceae family have been made. For instance, in *Cucumis melo* L., polyploids were successfully induced using oryzalin, where the obtained polyploid plants exhibited substantial morphological changes in seeds, leaves, and stomata compared with the diploid mother plants [18]. A similar study on the same species reported a higher total soluble solid content in tetraploid fruits than in diploid fruits [20]. In *Cucumis sativus* L., polyploids were produced using colchicine that displayed larger leaf size, flower diameter, stoma size, pollen grains, and more chloroplast numbers in guard cells [21]. In the same species, doubled haploid (DH) plants were obtained using colchicine, trifluralin, and oryzalin. The DH plants obtained exhibited larger leaves, flowers, and fruit sizes compared to their haploid relatives [16]. Polyploidy was also induced in *Citrullus lanatus* (Thunb.) Matsum and Nakai by colchicine treatment where the obtained polyploid plants had broader and thicker leaves of dark green color, and larger stomata, pollen grains, seeds, and fruits than their respective diploids [22,23]. Along with the morphological parameters, polyploidization could also influence the ratio of existing components in the phytochemical profile of the plants. For example, autotetraploidy in *Cichorium intybus* L. significantly influenced the concentrations of total phenolic compounds and chlorogenic acid in its leaves [24]. In *Thymus vulgaris* L., the essential oil yield and thymol content demonstrated notable increases [12], while the levels of macro- and micronutrients saw a rise in *Callisia fragrans* (Lindl.) Woodson [15].

Considering these developments in the Cucurbitaceae family, it could be hypothesized that artificial chromosome doubling could be an effective approach for obtaining genotypes with novel and desirable traits. Hence, the objective of this study was to obtain the autopolyploid plants in *M. scabra* Naudin by *in vitro* polyploidization using oryzalin as an antimitotic agent for the first time. This study also aimed to examine the influence of polyploidization events on the morphological, biochemical, and anatomical attributes in cucamelons. The findings obtained in this present study will serve as a foundation for future breeding attempts in this and related species. To the best of our knowledge, this was the first attempt at induced polyploidization in *M. scabra*.

8.2 Materials and methods

8.2.1 Plant material acquisition and *in vitro* transfer

Plantlets of *M. scabra* were sourced from the plant collection at the Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague, Czech Republic. *M. scabra* obtained was diploid with 12 basic number of chromosomes (x = 12) [25]. These plants were maintained in a 5 × 5 cm plastic pot within a greenhouse environment. The potting mix comprised sand, soil, peat moss, and vermiculite in a 1:1:1:1 ratio. The greenhouse maintained an average temperature of 23 °C, with relative air humidity levels fluctuating between 70% and 80%. Nodal segments were harvested from these maintained plants and subjected to surface sterilization. Surface sterilization included washing the nodal segments under running tap water for 10 min, followed by 1 min 70% ethanol wash and 10 min treatment with a 1% NaCl solution containing two drops of Tween 20. Finally, the nodal segments were rinsed thrice before transferring onto a basic Murashige and Skoog (MS) medium [26] without phytohormones. The explants were grown at 25/20 ± 0.5 °C under 16 h light/8 h darkness photoperiod. Illumination intensity was 3800 lux (51.3 µmol m^{-2} s^{-1}) from cool white fluorescent lamps. The plants were subcultured every 30 days until a sufficient number of plants were achieved for polyploid induction.

8.2.2 Autopolyploid induction

The polyploidy in *M. scabra* was induced using the nodal segments. The nodal segments were treated with 40, 60, and 80 µM of oryzalin for 24 and 48 h using the submersing method previously described [12]. The oryzalin solutions were prepared by dissolving an appropriate amount of oryzalin in a 1% DMSO (dimethyl sulfoxide) solution. A total of 40 nodal segments were treated in each concentration. After the treatment, the nodal segments were removed from the oryzalin solution, rinsed three times in sterilized distilled water, and cultivated individually in 100 mL Erlenmeyer flasks on the MS basal medium for the regeneration of the new shoots. Nodal segments subjected to basic MS medium without any oryzalin treatment was used as control variant. Three months after multiplication, the ploidy level was determined by a flow cytometer (Partec GmbH, Münster, Germany).

8.2.3 Flow cytometry analysis

The flow cytometry analysis was performed according to a previous study with slight modifications [12]. Approximately 1 × 1 cm^2 of leaf tissue was chopped using a sharp razor blade in a Petri dish containing 1 mL of Otto I buffer (0.1 M $C_6H_8O_7$ in 0.5% Tween 20). Crude

suspensions of the samples containing the isolated nuclei were subsequently filtered through a nylon mesh of 50 μm mesh size. The filtered supernatant was subjected to the dyeing step, where 1 mL of Otto II buffer (0.4M $Na_2HPO_4 \cdot 12$ H2O) containing fluorescent dye DAPI (2 μg/mL) was added to the filtered samples. All measurements to detect ploidy levels were executed using a Partec PAS flow cytometer (Partec GmbH, Münster, Germany), where at least 10,000 nuclei were recorded. Histograms of the relative DNA content were evaluated using the Flomax software package (Version 2.3). The stability of the ploidy level was retested after 10 months.

8.2.4 Transfer to *ex vitro* condition

For further evaluation of the morphophysiological parameters, two new autopolyploid genotypes (31 and 52) with stable ploidy levels were chosen, which showed good development and a great visual difference under *in vitro* culture conditions compared to the diploid genotype. The plants were selected and transferred to the greenhouse conditions where the average temperature was 23 °C with a relative humidity of 60–70%. The plantlets with well-developed root systems were removed from MS medium and transferred to plastic pots (5 × 5 cm) containing sand:soil:peat moss:vermiculite (1:1:1:1; *v*/*v*) mixture. The plants were maintained for 7 days covered with transparent polythene sheet under high humidity, and then the humidity was gradually lowered. The percentage of *ex vitro* survival was evaluated after 4 weeks.

8.2.5 Quantitative and morphological evaluation

In both autotetraploid and diploid (control) plants, various characteristics were assessed in the fruits, including weight, width, length, shape, and color. Seed attributes such as weight (weight of a hundred seeds), shape, and germination rates were also evaluated. Flower analysis involved examining size, petal count, and color. Leaves were assessed for both shape and color. All data were collected during the flowering stage and fruit harvesting period.

8.2.6 Determination of dry matter, ash, and crude protein content among the diploid and tetraploid fruits

Dry matter determination was performed based on two 5 g weights of each sample into pre-dried aluminum trays, which were then placed in an oven (Memmert, Germany) heated to 103 °C for 4 h. After drying, the trays were allowed to cool in a desiccator and then weighed.

The nitrogen content was evaluated according to the Kjeldahl method ISO 5983-1:2005 using a Kjeltec 2400 analyzer (FOSS, *Hilleroed,* Denmark). The crude protein content was calculated using a nitrogen-to-protein factor of 6.25. Ash contents were analyzed after mineralization of the fruits from all the genotypes at 550 °C in a muffle furnace (Nabertherm, Germany). The analyses of the parameters above were carried out in triplicate.

8.2.7 Determination of vitamin C, sugars, and citric acid among the diploid and tetraploid fruits

Preparation of samples for determination of vitamin C was carried out according to a previously described protocol [27]. To determine glucose, fructose, and citric acid, one gram of lyophilized sample was weighed into the 10 mL volumetric flask and 0.3 mL of Carrez reagent II (300 g of $ZnSO_4 \cdot 7H_2O$ in 1 L of demineralized water) was added, followed by 0.3 mL of Carrez reagent I (150 g of $K_4[Fe(CN)6] \cdot 3H_2O$ in 1 L of demineralized water). The prepared solution was topped up to the mark with demineralized water. Subsequently, the solution was centrifuged in a CompactStar CS 4 centrifuge (VWR, Leuven, Belgium) and the aqueous extract was filtered with a 0.45 µm PTFE filter (Agilent, Santa Clara, CA, USA).

A liquid chromatograph 1260 Infinity II (with vial sampler, pump, and column thermostat–MCT) (Agilent, Santa Clara, CA, USA) was used for the analysis. An Aminex HPX–87H Organic Acid Analysis Column 300 × 7.8 mm (Bio-Rad, Hercules, CA, USA) with RID detector (Agilent, Santa Clara, CA, USA) was used for sugars and a DAD WR detector (Agilent, Santa Clara, CA, USA) for citric acid analysis. A total of 20 µL of the sample was injected with a column flow rate of 0.6 $mL.min^{-1}$ with the heat of the thermostat 55 °C. A total of 0.005 M sulfuric acid solution was used as the mobile phase. A Poroshell 120 EC-C18 2.7 µm 3.0 × 150 mm column (Agilent, Santa Clara, CA, USA) and a DAD WR detector (Agilent, Santa Clara, CA, USA) were used for vitamin C analysis. For this analysis, 20 µL samples were injected and the column flow rate was 0.3 $mL.min^{-1}$ with the heat of the thermostat 25 °C. The mobile phase was composed of methanol and demineralized water, in a ratio of 40:760, and 0.2 µL of H_3PO_4 (Lachema, Brno, Czech Republic) was added to adjust the pH to 3. For each of the parameters, 3 biological and 3 technical replicates were used.

8.2.8 Total antioxidant activity comparison among the diploid and tetraploid fruits

A total of 1 g of the homogenized lyophilisate was weighed into 15 mL plastic centrifuge tubes and extracted with 10 mL of methanol overnight; the solubility of the substances was

supported with an ultrasonic water bath for 10 min. The samples were subsequently centrifuged (5 °C, 5 min, 3186 rcf). The supernatant was further diluted with methanol (1:1; *v*/*v*). Subsequently, 100 µL of the sample was added to 1.9 mL of methanolic DPPH solution (adjusted to absorbance A = 0.6 at 515 nm), and the contents were thoroughly mixed and left for 20 min at room temperature. Subsequently, the absorbance of the solution was measured at 515 nm (Thermo Fisher Scientific, Waltham, MA, USA). The samples were prepared in three parallel replicates and the total antioxidant activity was expressed as the average value in Trolox equivalent (TE) in µg/g sample dry weight. Three biological and three technical replicates were used.

8.2.9 Total phenolic content among the diploid and tetraploid fruits

The extract was prepared in the same way as for the determination of antioxidant activity. Next, 1 mL of extract was measured into 25 mL volumetric flasks, and 1 mL of Folin–Ciocalteu reagent and 3 mL of 20% aqueous sodium carbonate solution were added. The flasks were made up to 25 mL with demi-water and left for 2 h at room temperature. Subsequently, the absorbance at 765 nm was measured against a blank. The results were expressed as average values from three parallel repetitions in gallic acid equivalent (GAE) in µg/g dry weight of the sample. Three biological and three technical replicates were used.

8.2.10 Analysis of carotenoids among the diploid and tetraploid fruits

Briefly, the carotenoid-containing extract obtained from 0.5 g of lyophilized sample was subjected to alkaline hydrolysis with ethanolic potassium hydroxide at room temperature for 2 h. After liquid–liquid extraction of the hydrolysate with water and an ether:hexane mixture (1:1; *v*/*v*), the organic fraction was purified with water, concentrated, reconstituted with a suitable solvent, and analyzed by HPLC-DAD. The exact procedure for sample preparation, chromatographic separation, identification, and quantification of carotenoids is described in [28]. Three biological and three technical replicates were used.

8.2.11 Statistical analysis

Quantitative measurements were evaluated utilizing the Kruskal–Wallis test, a non-parametric method suitable for comparing more than two independent groups. A post hoc multiple comparison test of the mean rank order was conducted to scrutinize differences between specific groups further. For all analyses, a significance level of $p < 0.05$ was set to establish statistical significance, indicating substantial differences between the groups under investigation. The STATISTICA software package (Version 13.3) was used for all the analyses.

8.3 Results

8.3.1 Effect of oryzalin on the survival and polyploid induction rate

A total of 240 nodal segments were exposed to oryzalin across three concentrations and two time durations. The survival rate of explants was evaluated after 4 weeks. The survival rate varied from 20% to 70.5%, depending on the concentration and length of exposure of oryzalin to the explant. The explant survival rate was lower at a higher concentration and a longer exposure time (20%, at 80 µM and 48 h). On the other hand, at lower concentrations and shorter duration of exposure, the oryzalin was less toxic (Table 8.1). The surviving and regenerated plantlets were micropropagated, and after two months their ploidy levels were determined by flow cytometry analysis (FCM). A total of 20 autotetraploid and 15 mixoploid plants were detected (Table 8.1). Figure 8.1a,b show histograms generated by FCM depicting the relative DNA content among diploid and tetraploid plants. Samples with multiple peaks were considered as mixoploids (image not shown). Polyploidization efficiency of oryzalin in *M. scabra* ranged between 2.5% and 15% (Table 8.1). The highest number of polyploids was obtained in the treatment where explants were treated with 80 µM oryzalin for 48 h (six tetraploids).

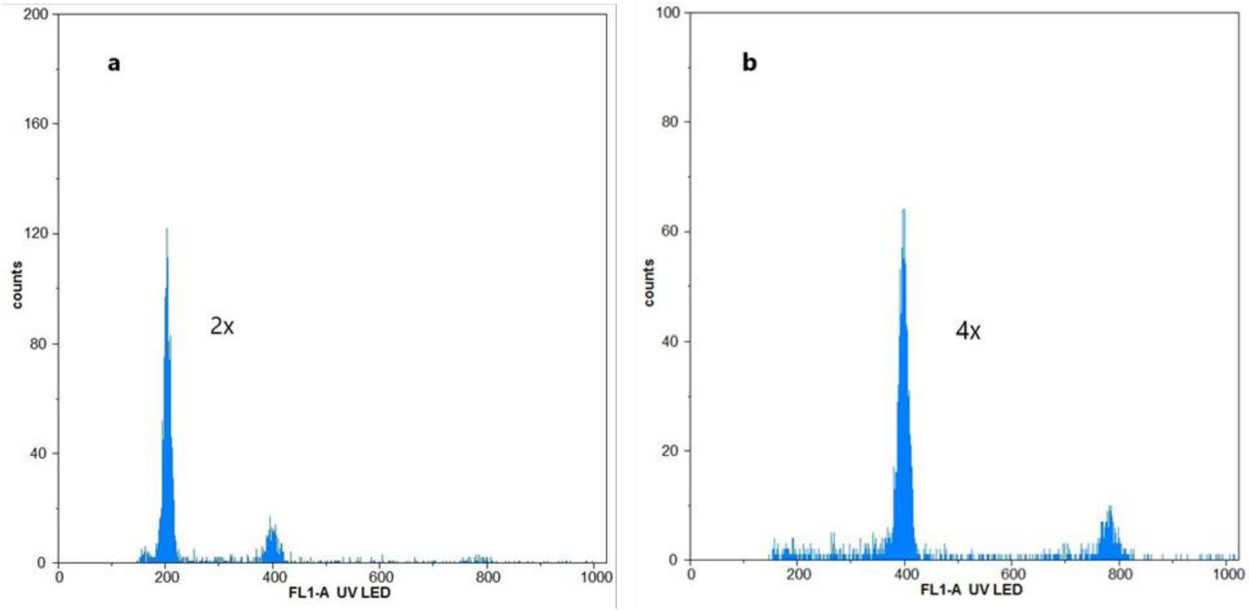

Figure 8.1 Histograms of flow cytometry analysis from *M. scabra*: (a) control plant (diploid) and (b) polyploid plants (tetraploid).

Table 8.1 Effect of polyploidization on nodal segments of *M. scabra* Naudin using oryzalin.

Treatment	Oryzalin (µM)	Number of treated explants	Time of treatment (h)	Survival rate (%)	Number of tetraploid plants	Number of mixoploid plants	Polyploidization efficiency of tetraploids (%)
T1	40	40	24	70.5	3	3	7.5
T2	40	40	48	62.5	3	1	7.5
T3	60	40	24	60.0	3	4	7.5
T4	60	40	48	50.0	4	3	10.0
T5	80	40	24	37.5	1	2	2.5
T6	80	40	48	20.0	6	2	15.0

8.3.2 Morphological comparisons between diploid and tetraploids of *Melothria scabra*

The plants were cultivated in greenhouse conditions. Diploid and polyploid plants had vegetation periods of 120 days and 155 days, respectively. The flowering time among the polyploid genotypes differed, with the first flowers in diploid plants observed after 50 days of cultivation and in polypoid plants after 57 (for genotype 31) and 60 (for genotype 52) days of cultivation. The flowers from diploid and tetraploid genotypes varied significantly (Table 8.2 and Figure 8.2). The flowers varied in the width of the entire flower. Polyploid plants had larger flowers (36% on average) with the same number of petals as diploid plants (Table 8.2).

Figure 8.2 Morphological variation between diploid (a) and induced tetraploid (b) flowers of *M. scabra* cultivated in greenhouse conditions.

The development of fruits till maturity took, on average, 17 days in control and 20 and 25 days in genotypes 52 and 31, respectively, from the beginning of flowering. There was a significant difference in the length, width, and weight of the fruit (Table 8.3 and Figure 8.3). Fruits of polyploid plants (genotype 52) were on average 10% shorter than those of diploid plants, but all polyploid plants had wider (13–15%) fruit with significantly higher weight (14–23%).

Table 8.2 Morphological evaluation of the flower from control and polyploid genotypes.

Variant	Flower width (mm)	Receptacle width (mm)	Flower height (mm)	Number of petals
Control	8.46 ± 1.74 a	2.28 ± 0.71 a	2.89 ± 0.57 a	5 ± 0 a
Genotype 31	11.18 ± 1.24 b	2.83 ± 0.35 a	3.05 ± 0.64 a	5 ± 0 a
Genotype 52	11.5 ± 1.29 b	2.69 ± 0.57 a	3.33 ± 0.45 a	5 ± 0 a

Different letters within the same column differ significantly. Data were tested by Kruskal–Wallis test with a *p*-value of 0.05.

Figure 8.3 Morphological variation between diploid (2×) and induced tetraploid (genotypes 31 and 52) fruit of *M. scabra*.

However, when the seeds obtained from the fruits were compared, control fruits had the longest seeds (3.1 cm), the widest seeds (1.7 cm), and the highest average weight of 100 seeds (375.25 mg). In contrast, both polyploid genotypes 52 and 31 exhibited shorter and narrower seeds with significantly lower average seed weights (Table 8.3 and Figure 8.4). Additionally, the induced polyploids failed to germinate, whereas the diploid seeds had a germination rate of 85%. Tetraploid induction also had a significant effect on morphological traits of the leaf (Figure 8.5). The leaf blade margins of polyploid plants were more dentate than those of diploid plants. Changes in the color of the leaves were also distinctly different. The leaves of genotype 31 had a light green coloration, and genotype 52 showed a dark green coloration compared to their diploid forms.

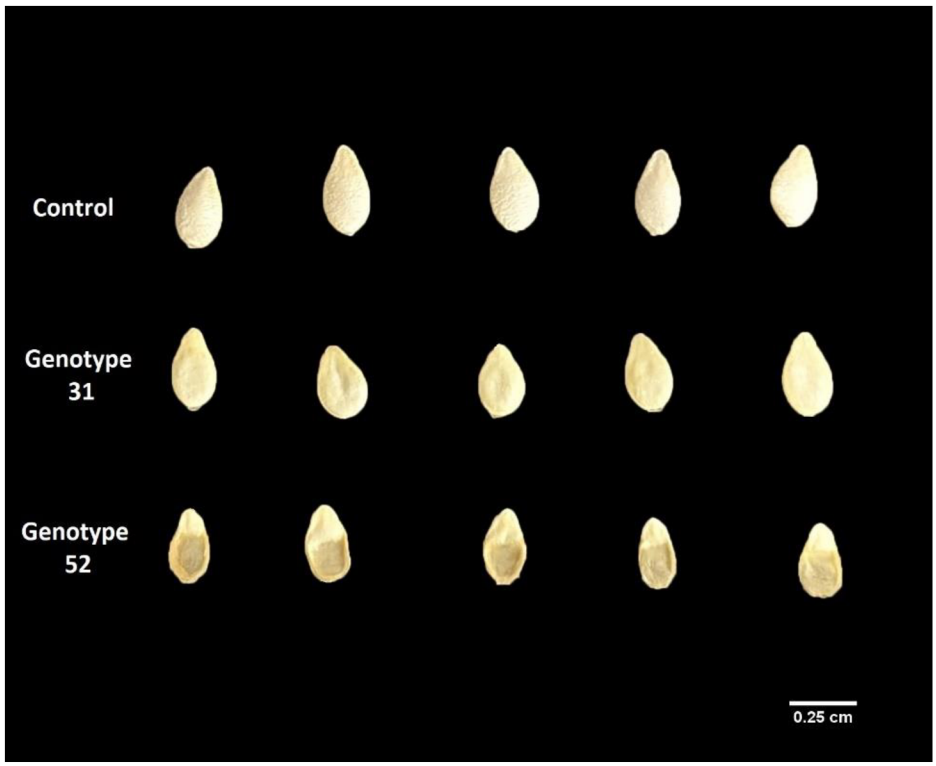

Figure 8.4 Comparison of seed morphology among diploid and polyploid genotypes.

Table 8.3 Morphological evaluation of the fruits and seeds from control and polyploid genotypes.

Variant	Fruit length (mm)	Fruit width (mm)	Fruit weight (g)	Seed length (mm)	Seed width (mm)	Average weight of 100 seeds (mg)
Control	27.1 ± 1.63 a	14.9 ± 0.64 a	3.4 ± 0.45 a	3.1 ± 0.1 a	1.7 ± 0.04 a	375.2 ± 27.5 a
Genotype 31	27.7 ± 2.42 a	17.0 ± 0.97 b	4.2 ± 0.58 b	2.9 ± 0.1 ab	1.8 ± 0.09 a	83.3 ± 7.5 b
Genotype 52	24.5 ± 2.01 b	17.2 ± 0.91 b	3.9 ± 0.39 b	2.7 ± 0.1 b	1.5 ± 0.01 b	76.2 ± 5.8 c

Different letters within the same column differ significantly. Data were tested by Kruskal–Wallis test with a *p*-value of 0.05.

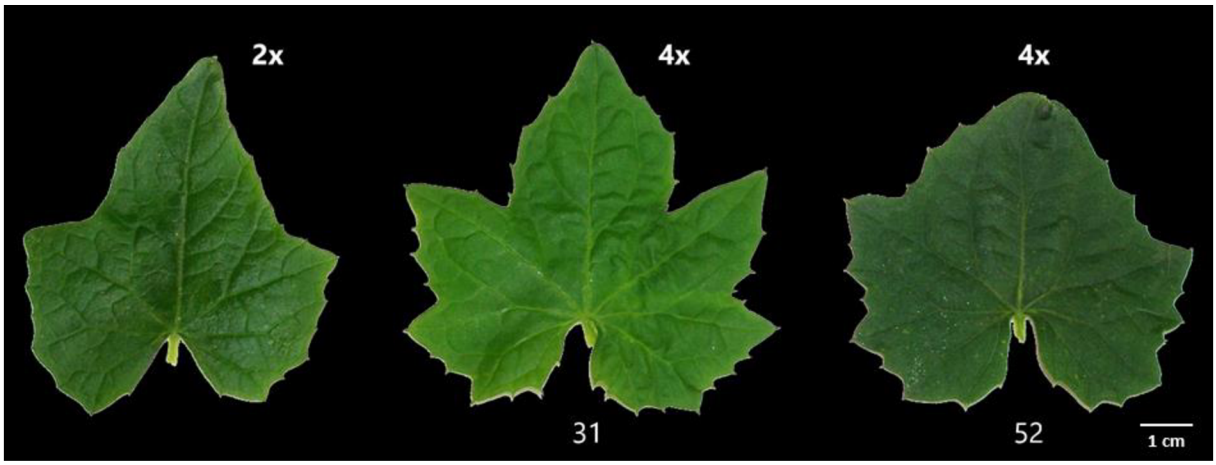

Figure 8.5 Morphological variation between diploid (2×) and induced tetraploid (4×) (genotypes 31 and 52) leaves of *M. scabra*.

8.3.3 Nutritional comparisons between diploid and autotetraploids of *Melothria scabra*

Ripe fruits were collected and analyzed immediately after harvesting within a few hours. All fruits were harvested and processed under the same conditions. In this comparative study, we assessed the polyploid genotypes (genotypes 31 and 52) and compared them to the control genotype for nutritional and physiological parameters. The findings revealed significant

differences among the genotypes for several key parameters. The control genotype exhibited the highest dry matter content (10.30 g/100 g fresh weight (FW)), crude protein content (21.15 g/100 g DM), ash content (4.19 g/100 g DM) and vitamin C content (14.49 mg/kg DM), while genotypes 31 and 52 generally showed lower values in these categories. However, genotypes 31 and 52 had significantly higher glucose and fructose content than the control genotype (Table 8.4). Citric acid was significantly similar in the control (15.26 g/100 g DM) and in genotype 52 (13.50 g/100 g DM), whereas genotype 31 (11.35 g/100 g DM) exhibited lower values. These results highlight significant variations in nutritional and physiological parameters among the polyploid and the control genotypes, emphasizing the potential impact of polyploidization on these characteristics.

Table 8.4 Nutritional evaluation of the fruits from control and polyploid genotypes of *M. scabra* Naudin.

Variant	Dry weight g/100 g FW	Crude protein g/100 g DM	Ash g/100 g DM	Vitamin C mg/kg DM	Glucose mg/100 g DM	Fructose g/100 g DM	Citric acid g/100 g DM
Control	10.30 ± 0.07 a	21.15 ± 0.88 a	4.19 ± 0.07 a	14.49 ± 0,71 a	109.67 ± 3.04 c	8.30 ± 0.18 c	15.26 ± 0.72 a
Genotype 31	7.98 ± 0.05 b	15.74 ± 0.60 b	3.25 ± 0.06 b	12.77 ± 0.66 b	189.28 ± 0.75 b	12.84 ± 0.01 b	11.35 ± 0.19 b
Genotype 52	7.73 ± 0.12 b	16.23 ± 0.62 b	3.16 ± 0.03 b	11.96 ± 0.20 b	265.65 ± 0.75 a	14.22 ± 0.01 a	13.50 ± 0.01 a

Different letters within the same column differ significantly. Data were tested by Kruskal–Wallis test with a *p*-value of 0.05. DM = dry matter, FW = fresh weight.

8.3.4 Polyphenol, antioxidant, and carotenoid Contents

The results revealed notable differences in these biochemical parameters among the control fruits and the fruits from the induced polyploid genotypes, genotype 31 and genotype 52. The control group exhibited a polyphenol content of 1183 µg GAE/g. Notably, both genotype 31 and genotype 52 displayed significantly higher polyphenol content, with values of 2055 µg GAE/g and 2377 µg GAE/g, respectively. Conversely, the antioxidant activity of all the variants did not differ, where the control group exhibited an antioxidant content of 1509 µg TE/g. Genotype 31 and genotype 52 had a comparable antioxidant content of 1467 µg TE/g and 1527

μg TE/g, respectively (Table 8.5). The carotenoid content in the fruits was further analyzed by measuring the concentrations of specific carotenoids, including lutein, zeaxanthin, α-carotene, and β-carotene. Notably, genotype 52 exhibited a substantial increase in lutein content, recording a value of 68.2 μg/g DW compared to the control (62.61 μg/g DW) and genotype 31 (44.63 μg/g DW). Additionally, genotype 52 also displayed a significantly higher zeaxanthin content (3.46 μg/g DW) compared to the control (2.89 μg/g DW) and genotype 31 (2.48 μg/g DW). However, no significant differences were observed in the levels of α-carotene and β-carotene among the three groups.

In this study, the polyploid genotypes (genotypes 31 and 52) were compared with the control genotype for the content of carotenoids. The control genotype had the highest lutein content (62.61 μg/g), which was significantly different from genotype 31 (44.63 μg/g) and genotype 52 (68.2 μg/g). Zeaxanthin content did not significantly differ among the genotypes, with values ranging from 2.48 to 3.46 μg/g. Genotype 31 exhibited the highest α-carotene content (0.87 μg/g), which was significantly different from the control genotype (0.72 μg/g) and genotype 52 (0.84 μg/g). The β-carotene content did not show significant differences among the genotypes, with values ranging from 18.34 to 21.93 μg/g.

Table 8.5 Comparison of polyphenols, antioxidant activity, and carotenoids content in fruits of *M. scabra* Naudin and its induced polyploids.

Variant	Polyphenols (μg GAE/g DW)	Antioxidant activity (μg TE/g DW)	Carotenoids (μg/g DW)			
			Lutein	Zeaxanthin	α-Carotene	β-Carotene
Control	1183 ± 26.66 c	1509 ± 42.00 a	62.61 ± 5.60 a	2.89 ± 0.29 b	0.72 ± 0.24 a	18.46 ± 4.85 a
Genotype 31	2055 ± 14.04 b	1467 ± 49.35 a	44.63 ± 2.04 b	2.48 ± 0.04 b	0.87 ± 0.11 a	18.34 ± 2.86 a
Genotype 52	2377 ± 22.27 a	1527 ± 17.95 a	68.2 ± 9.63 a	3.46 ± 0.40 a	0.84 ± 0.25 a	21.93 ± 5.79 a

Different letters within the same column differ significantly. Data were tested by Kruskal–Wallis test with a *p*-value of 0.05.

8.4 Discussion

Polyploidization is a natural biological process that can result in novel genetic material, giving rise to unique characteristics distinct from those found in the progenitor forms. Typically, polyploid organisms demonstrate enhanced traits with increased resilience to both biotic and abiotic stresses compared to their diploid counterparts [17]. Artificially, polyploidization as a breeding tool has long been used for the improvement of numerous plant species [9–13,15,29]. It is a fast, cost-effective, and reliable approach for introducing novel genotypes with desirable traits [9]. Oryzalin was used as an antimitotic agent in this study as a polyploid induction agent in *M. scabra*. The efficiency of polyploidization achieved using oryzalin reached up to 15%. Similar to the current study, a polyploidization rate of up to 11.11% was obtained in oriental melon when oryzalin was used as an antimitotic agent [20].

Morphological parameters are often used as the first primary screening criteria for polyploids but are generally not completely reliable. Hence, chromosome counting is considered as the most direct and accurate method to identify polyploids [30]. However, this approach has several drawbacks, including that it is time-consuming and laborious. It often needs to be optimized for individual plant species, and often the counting of smaller chromosomes leads to inaccuracies [9,30,31]. In contrast, flow cytometry is a fast and reliable approach to identifying polyploid individuals. This technique eliminates the need for labor-intensive chromosome counting, providing a rapid and reliable assessment of ploidy levels [31]. In the current study, the flow cytometric analysis employed was effective in screening out the polyploids from the treated population. While chromosome counting is a valuable complementary method, its omission did not compromise the accuracy of polyploid identification through flow cytometry.

It was elucidated from the current study that artificial polyploidization in *M. scabra* triggers gigantism, where the polyploid plants displayed significantly larger leaves, flowers, and fruits. The phenomenon of gigantism caused by polyploidization has been frequently reported in plants from Cucurbitaceae and other families. For instance, in *C. lanatus* [22,23] and *C. melo* [18], induced tetraploid plants exhibited significantly larger flowers, leaves, seeds, and fruit size than diploid plants. However, a study by Bae et al. [17] was not in line with these findings for tetraploid plants of *C. lanatus*, and the induced tetraploid plants showed small, thick, and crumpled leaves. This discrepancy suggests that the effects of polyploidization on plant morphology may vary depending on the type of plant species employed. One crucial finding from the current study was observed in the seed characteristics. The induced polyploid plants

exhibited a notable decrease in the average seed weight, suggesting inadequately filled endosperm. This could potentially explain the observed failure in germination, as the seeds might lack the required nutrient reserves for germination. Further molecular and physiological investigations could provide deeper insights into the underlying mechanisms. While polyploid genotypes with chaffy seeds or kernels could pose challenges in terms of seed germination and crop establishment, the polyploid minimelon fruits with chaffy seeds could have positive implications from a consumer perspective.

The primary agronomical gain from this plant is the striped fruit, which is sweet and resembles a watermelon. The effect of polyploidization on the fruit characteristics constitutes an essential aspect from the breeding point of view. Several key parameters, including dry weight, ash weight, wet weight, vitamin C, glucose, fructose, and citric acid content, were systematically examined in the current study. The fresh weight of the fruits from the polyploid genotype displayed significantly higher values (14–23%). A higher weight of fruits can directly translate into a higher yield of a fruit crop. While the wet weight of the fruits increased significantly, the dry weight and the ash weight of the polyploid fruit were about 22–25% less than in the control. This indicates that the polyploid fruits had higher water content. Noteworthily, the polyploidization positively influenced the glucose and fructose content of the fruits from both genotypes. Glucose content increased by 73% in genotype 31 and 142% in genotype 52 compared to the control variant. Similarly, the fructose content increased by 55% in genotype 31 and 71.32% in genotype 52 compared to the control variant. This is a significant finding, as increased sugar content can enhance the sweetness and palatability of the fruits, potentially making them more appealing to consumers. Similar results where induced polyploid genotypes demonstrated higher fruit weight and elevated sugar contents of the fruits have been previously reported [32–34]. These enhancements could be attributed to the changes in gene expression, involving both the upregulation and downregulation of processes related to biosynthesis, transport, reception of primary and secondary metabolites, and various enzymes [34,35]. Overall, these findings highlight the potential of synthetic polyploidization in improving desired fruit traits.

In this study comparing a control genotype with two induced polyploid genotypes, several notable differences in biological activity were observed. Specifically, polyphenols were present in higher amounts in the polyploid genotypes compared to the control. Polyphenols are known to have numerous health benefits, including protection against cardiovascular diseases, diabetes, insulin resistance, and certain cancers [36,37]. Polyphenols can also act as antioxidants to fight against the oxidative damage caused to the cells [37]. While the

polyphenols were higher in the polyploids, the levels of antioxidant activity among the polyploid and diploid genotypes were not significantly different. Despite having higher polyphenols, the comparable antioxidant activity among polyploid genotypes and the control genotype could be attributed to the higher amount of vitamin C (13–21%) present in the control genotype compared to the fruits from the polyploid genotypes. Vitamin C is a well-known antioxidant found in plants, vegetables, and fruits [38]. Carotenoids are natural nutrients found in fruits and vegetables that possess numerous health benefits [39]. For example, a higher lutein intake can help to remedy conditions related to eyesight, like cataracts [40]. To the best of our knowledge, the current study assessed the carotenoids, namely lutein, zeaxanthin, α-carotene, and β-carotene, for the first time in *M. scabra*. Lutein and β-carotene were found to be present in major portions in the fruits of the control and polyploid genotypes. The polyploid genotypes displayed a very similar profile to that of the diploid for the assessed carotenoids except for zeaxanthin in genotype 52, which had significantly higher value, and lutein, where genotype 31 exhibited lower values. Overall, the results suggest that induced polyploidy, particularly in genotype 52, can significantly impact the biological activity of these genotypes, offering potential applications in agriculture and nutrition.

Another advantage of polyploidy is that it leads to novel genotypes resistant to various biotic and abiotic stresses [41]. There are studies that report that polyploid plants have a greater resistance to biotic and abiotic stress factors. For example, in apple (*Malus x domestica* Borkh), the resistance of autotetraploids to *Venturia inaequalis* [42], *Alternaria alternata*, and *Colletotrichum gloeosporioides* [43] infection is reported in comparison to their diploid forms. Similar results were presented for *Anemone sylvestris*, where the autotetraploids had a better response to *Phytophthora plurivora* infection [10]. In the case of *M. scabra* in the current study, the diploid (control) plants were observed to have a fungal infection of cucurbit powdery mildew (*Sphaerotheca fuliginea* and *Erysiphe cichoracearum*). On the other hand, the polyploid plants were only slightly attacked by these fungi, even though they were growing in proximity to the diploid plants. Considering these previous findings and preliminary observations from the current study, it would not be baseless to assume that polyploid plants of *M. scabra* could be more tolerant to biotic factors compared to their diploid forms. However, a systematic study to confirm this hypothesis needs to be carried out. The research on the effects of polyploidy on biotic and abiotic factors could help to obtain new genotypes of horticultural crops with greater resistance, and thus obtain high quantitative and qualitative yields.

8.5 Conclusions

In summary, our study successfully induced polyploidy in *M. scabra* through the application of oryzalin, resulting in substantial morphological and biochemical transformations. The induced autotetraploid plants exhibited distinct vegetative and reproductive characteristics, including enlarged flowers, increased fruit weight, and altered leaf morphology. Nutritional analysis unveiled variations in key parameters, including an elevation in sugar levels in polyploids, underscoring the potential of polyploidization to enhance nutritional content. Moreover, the generated polyploid genotypes demonstrated enhanced biological activities. These comprehensive findings provide valuable insights into the prospective applications of polyploidy for crop enhancement, emphasizing improved nutritional quality in cucamelons and related crops. Future studies should be aimed to assess these novel genotypes for their resilience to both abiotic and biotic stresses, further advancing our understanding and potential utilization of polyploidization in agricultural contexts.

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9. Polyploidization and genomic selection integration for grapevine breeding: a perspective

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This chapter explores the status of chromosome doubling attempts in Vitis spp., and addresses the potential challenges associated with the polyploid breeding in Vitis spp. Further, this chapter also presents a novel strategy to overcome associated challenges through the integration of genomic selection and multiomics approaches.

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Abstract

Grapevines are economically important woody perennial crops widely cultivated for their fruits that are used for making wine, grape juice, raisins, and table grapes. However, grapevine production is constantly facing challenges due to climate change and the prevalence of pests and diseases, causing yield reduction, lower fruit quality, and financial losses. To ease the burden, continuous crop improvement to develop superior grape genotypes with desirable traits is imperative. Polyploidization has emerged as a promising tool to generate genotypes with novel genetic combinations that can confer desirable traits such as enhanced organ size, improved fruit quality, and increased resistance to both biotic and abiotic stresses. While previous studies have shown high polyploid induction rates in *Vitis* spp., rigorous screening of genotypes among the produced polyploids to identify those exhibiting desired traits remains a major bottleneck. In this perspective, we propose the integration of the genomic selection approach with omics data to predict genotypes with desirable traits among the vast unique individuals generated through polyploidization. This integrated approach can be a powerful tool for accelerating the breeding of grapevines to develop novel and improved grapevine varieties.

9.1 Introduction

Grapevines (*Vitis* spp.) are woody perennial crops belonging to the Vitaceae family. These are extensively cultivated for their fruits, which are used in wine production, along with for grape juice, raisins, and table grapes. The wine industry has a substantial influence on the global economy. Additionally, grapes also contain beneficial compounds, such as resveratrol and flavonoids, which have been shown to have antioxidant, anti-inflammatory, and anti-cancer properties and help prevent chronic diseases (Sabra et al., 2021; Zhou et al., 2022; Cuciniello et al., 2023). Currently, grapevine breeding is facing several environmental challenges such as unforeseen climate change and pervasiveness of diseases and pests (Marín et al., 2021; Töpfer and Trapp, 2022). On the other hand, pests and diseases lead to substantial yield losses and abridged fruit quality. To overcome these challenges, grapevine breeders develop drought-tolerant or disease-resistant varieties. However, producing these varieties via traditional breeding methods can be an extensive, lengthy, and complex process. One possible alternative to these traditional breeding methods can be artificial polyploidization. In the context of grapevine breeding, artificially increasing the number of sets of chromosomes and creating a polyploid can be a promising tool to generate genotypes with novel genetic combinations not present in the parental lines. Polyploidization can confer agronomically desirable traits, such as enhanced organ size, improved fruit quality, and increased resistance to both biotic and abiotic stresses (Touchell et al., 2020; Gantait and Mukherjee, 2021; Beranová et al., 2022; Jin et al., 2022; Bharati et al., 2023). Furthermore, this method offers a range of advantages over traditional breeding techniques, such as rapid production of polyploid individuals, increased genetic diversity, cost-effectiveness, and applicability across a broad spectrum of plant species.

While synthetic polyploidization has proven to be a potent tool in breeding various plants, its full potential in grapevine breeding remains untapped. However, there are some plants such as *Anemone sylvestris* (Šedivá et al., 2019), *Thymus vulgaris* (Homaidan Shmeit et al., 2020), and *Lycium ruthenicum* (Rao et al., 2020), where polyploidation has been used previously. Synthetic polyploidization can quickly generate a high frequency of polyploids, however, it necessitates meticulous genotype screening to screen for desired traits. Genotype screening after polyploidization may be more straightforward for crops with shorter life cycles or those that exhibit early expression of desired traits, such as herbs. However, when it comes to perennial crops like grapevines, this process demands substantial labor and financial investments. This could potentially explain the limited research on screening genotypes with desired agronomic

traits, such as increased yield and tolerance to abiotic and biotic stress, after polyploidization in any *Vitis* species.

The genomic selection (GS) of the produced polyploids can be an interesting option, while predicting the desirable genotypes following the artificial polyploidization. In general, GS involves using genomic information to predict the breeding value of plants and selecting the best individuals with desired traits of interest for further breeding (Newell and Jannink, 2014; Bhat et al., 2016; Crossa et al., 2017). In the context of grapevine breeding, the breeders can easily envisage the genetic potential of an individual polyploid plant for a given trait, bypassing the time-consuming and labor-intensive screening methods. In the current perspective, we will discuss the current state of the polyploidization in the grapevines towards crop improvement. Additionally, we aim to identify the potential of polyploidization and GS integration towards predictive breeding of grapevines. This integrated approach can be a powerful tool for accelerating the breeding of grapevines to develop novel and improved grapevine varieties. This will not only help breeders obtain genotypes with high agronomic value but will also reduce the time, labor, and capital investments that would otherwise become futile if poorly performing genotypes are obtained.

9.2 Polyploidization in grapevine improvement: current status and limitations

Polyploidization has been used as a tool for crop improvement for many years. To date, this technique has been successfully used in many species to obtain traits such as increased fruit size, enhanced disease resistance, and tolerance to a variety of stresses (Šedivá et al., 2019; Homaidan Shmeit et al., 2020; Rao et al., 2020). Recent studies have focused on inducing polyploids in *Vitis* through various methods and anti-mitotic agents. *In vivo* methods, which involve treating the entire plant or a part of a plant, have been attempted for polyploidization in *Vitis* species, but have remained less effective compared to *in vitro* methods. For example, in a study by Kara et al. (2018), the use of colchicine treatment in grape genotypes resulted in no tetraploid plants being identified, except for one grape cv (Trakya İlkeren), which showed aneuploidy at a specific concentration of colchicine. Similarly, in another study by (Kara and Doğan, 2022), the application of oryzalin and N2O to cuttings of 41B Chasselas and Fercal (*Vitis vinifera* L.) rootstocks did not result in the production of any polyploid individuals through *in vivo* methods. Kara and Yazar (2021) also examined changes in stomata guard cells but found no differences at the ploidy level. More recently, Kara and Doğan (2023) utilized

Oryzalin and N_2O to treat a total of 1200 plants belonging to two grapevine cultivars, yielding only one tetraploid genotype for each cultivar. These results suggest that *in vivo* methods for polyploidization in *Vitis* species are not an effective approach in obtaining high frequency polyploids.

Recent progress with *in vitro* methods offers a promising avenue for inducing polyploids in many *Vitis* species. Acanda et al. (2015) found that the *in vitro* treatment of colchicine at a concentration of 0.2% was most effective for producing tetraploid plantlets in *Vitis*, with a tetraploid rate of 25%. Xie et al. (2015) also achieved successful polyploidization in *Vitis* through colchicine treatment, with a polyploid induction rate of 37.78%, when pre-embryogenic calli were used. Additionally, Sinski et al. (2014) found that both colchicine and oryzalin were effective in inducing polyploids in *Vitis*. Although, oryzalin was found to be more effective than colchicine in polyploid induction efficiency in *Vitis* spp., ranging between 1.66-10.5% compared to 3.2-5% (Table 9.1). These recent developments in polyploidization techniques in *Vitis* species could have significant implications for improving crop yield and quality in viticulture. Interestingly, polyploidization in *Vitis* species under *in vitro* conditions without the use of anti-mitotic agents has also been observed. Catalano et al. (2021) regenerated grapevine plants via somatic embryogenesis and observed a 9% tetraploid induction rate, even though no anti-mitotic agents were used to induce polyploidization. The overall findings of the studies suggest that *in vitro* chromosome doubling could be a viable approach to generate polyploid grapevines with desirable characteristics, which could potentially have significant implications for the grape industry. A list of major attempts to induce polyploids in a number of *Vitis* species has been summarized in Table 9.1.

Table 9.1 List of major artificial polyploidization attempts in various *Vitis* Spp.

Reference	*Vitis* species	Anti-mitotic agent used	Mode of treatment	Findings
Kara and Doğan, 2023	41 B [Chasselas (*Vitis vinifera* L.) × (*Vitis* berlandieri Planch.)] and Fercal [(*Vitis vinifera* L. × *Vitis berlandieri*) × 333 EM (Cabernet-Sauvignon × *Vitis berlandieri*)]	Oryzalin and N_2O	*in vivo*	A total of 1200 plants for each genotype and each anti-mitotic agent were used for polyploid induction. For the 41 B genotype, one mixoploid plant and one tetraploid plant were obtained. For the Fercal genotype, four mixoploid plants and one tetraploid plant were obtained.
Kara and Doğan, 2022	41 B Chasselas (*Vitis vinifera* L. × *Vitis berlandieri* Planch) and Fercal [(*Vitis vinifera* x *Vitis berlandieri*) × 333 EM]	Oryzalin and N_2O	*in vivo*	The application of oryzalin and N2O to cuttings of 41B and Fercal rootstocks did not result in the production of any polyploid individuals through *in vivo* methods.
Kara and Yazar, 2022	*Vitis vinifera* L. (Ekşi Kara & Trakya İlkeren)	Colchicine (were applied to meristematic part of seedling twice a day (in 8.30 and 18:00) for 3 days)	*in vivo*	The viability of shoot tips varied among the cultivars and decreased with increasing colchicine doses, except for the application of 1 g L-1 on 'Ekşi Kara' and 5 g L-1 on 'Trakya İlkeren'. In 'Ekşi Kara', the lowest shoot tip viability rates compared to the control (100%) were observed at doses of 4 g L-1 (31.77%), 6 g L-1 (47.26%), and 3 g L-1 (51.84%). Colchicine was administered to seedlings from two grape cultivars, resulting in polyploidy induction, depending on the application methods and genotypes. 5 mg/L was found to be effective for Ekşi Kara and 6 mg/L was effective for Trakya İlkeren seedlings.
Kara and Yazar, 2021	*Vitis vinifera* L.	Colchicine	*in vivo*	Examination through chloroplast counts and FC analyses of stoma guard cells revealed that these changes did not result in any differences at the genomic level.
Catalano et al., 2021	*Vitis vinifera* L. (Catarratto, Frappato, and Nero d'Avola)	none	*in vitro*	Grapevine plants regenerated via somatic embryogenesis in this study observed a nine percent tetraploid induction rate.

Kara and Yazar, 2020	Ekşi Kara (*Vitis vinifera* L.)	Colchicine	*in vivo*	Eight different colchicine concentrations (0, 1, 2, 3, 4, 5, and 7.5 g L-1) were administered twice daily (at 8:30 AM and 6:00 PM) to the meristematic part of seedlings for a duration of 3 days, starting when the first true leaves appeared. Although, flow cytometric analysis confirmed that no polyploids were obtained indicating the employed approach was ineffective.
Kara et al., 2020	41 B Chasselas (*Vitis vinifera* L. × *Vitis berlandieri* Planch) and 'Trakya İlkeren', ' Gök Üzüm' and 'Ekşi Kara' grape cultivars (*Vitis vinifera* L.)	N_2O	*in vivo*	Flow cytometric analysis confirmed that the application of N_2O failed in polyploidy induction in grapevine genotypes used.
Kara et al. 2018	41 B Chasselas (*Vitis vinifera* L.) × (*Vitis berlandieri* Planch.), Gök Üzüm (*Vitis vinifera* L.), Trakya İlkeren (*Vitis vinifera* L.)	Colchicine	*in vivo*	The use of colchicine treatment in grape genotypes used by authors revealed that all untreated seedlings had diploid ploidy levels (2n=2x=38), and no tetraploid plants were identified. Only the grape cv Trakya İlkeren responded to the colchicine treatment, inducing aneuploidy at a concentration of 5 gL-1, resulting in a ploidy level of 2n=2x=40.
Xie et al., 2015	*Vitis* × *Muscadinia*	Colchicine and oryzalin	*in vitro*	This research accomplished the successful generation of a significant proportion of tetraploid plants from hybrids of 101-14 Mgt X *M. rotundifolia* cv. Trayshed. Colchicine treatment was found most effective, with the highest polyploid induction rate of 37.78% when pre-embryogenic calli were used for treatment.
Acanda et al., 2015	*Vitis vinifera* L. cv. Mencı´a	Colchicine	*in vitro*	In this study, the most effective concentration of colchicine for producing tetraploid plantlets was found to be 0.2%, resulting in a tetraploid rate of 25%. No mixoploid or chimeric plantlets were observed during the experiment.

Chang et al. 2014	Victoria grape (*Vitis vinifera* L.)	Colchicine	*in vitro*	The most effective method to enhance chromosome duplication efficiency was observed by treating the third and fourth buds with 0.05% colchicine for 48 hours or 0.1% colchicine for 24 hours. The primary generation cells displayed doubling rates of 33% and 31% respectively.
Sinski et al., 2014	*Vitis vinifera* L. (Crimson seedless and BRS Clara)	Colchicine and oryzalin	*in vitro*	In this study, colchicine and oryzalin were both effective in inducing polyploids. Oryzalin was found to be more effective in polyploid induction efficiency ranging between 1.66-10.5%, than colchicine with 3.2-5%.
Yang et al., 2006	*Vitis vinifera* L. cv. Sinsaut	Colchicine	*in vitro*	A total of 29 plantlets generated from embryos treated with colchicine were examined. Out of the 29 plantlets, five (which constituted 17.2%) were found to be tetraploid (2n = 2x = 76), while all the remaining plantlets were diploid (2n = 2x = 38). The application of colchicine to somatic embryos did not result in the production of any chimeras.
Notsuka et al., 2000	*Vitis vinifera* L. and American hybrids	Colchicine	*in vitro*	Axillary buds of growing shoots were used to perform *in vitro* chromosome doubling on 29 diploid, 3 triploid, and 1 tetraploid grape accession of *Vitis* spp. The success rates varied among the accessions, with the range being from 6% (for 'Hakata White') to 47% (for 'Pusa Seedless') in *V. vinifera*, and from 4% (for 'Fuefuki') to 35% (for 'Prima Seedless') in the American species.

While the successful induction of polyploids in grapevines has been documented in several studies, only a few have assessed the resulting population for desirable agronomical traits, where these assessments have primarily focused on stomatal and leaf characteristics (Yang et al., 2006; Sinski et al., 2014; Xie et al., 2015; Kara et al., 2018; Kara et al., 2020; Catalano et al., 2021; Kara and Yazar, 2021; Kara and Doğan, 2022; Kara and Yazar, 2022). A study has also delved into epigenetic regulation through DNA methylation, shedding light on how changes in DNA methylation patterns can impact gene expression and phenotypic traits in polyploid grapevine (Xiang et al., 2023). Surprisingly, the essential agronomical traits with economic value, including vigor, yield, berry size, berry color, Brix levels, as well as ripening period, have received minimal attention in this context. One notable exception is the study by Notsuka et al. (2000), which comprehensively evaluated the generated polyploids with a specific emphasis on grape-related traits, recognizing that fruit-related traits require a more substantial investment of time and effort. In this study, the authors explored the potential of *in vitro* chromosome doubling across 29 diploid, 3 triploid, and 1 tetraploid grape accession of *Vitis* spp., successfully achieving high polyploid inductions of up to 47%. Subsequent field trials of these polyploids unveiled a diverse range of desirable traits, including vigorous growth, improvements in skin color, and enhanced berry size. However, it is noteworthy that the performance of induced polyploids varied significantly depending on the cultivar. In some cases, the induced polyploids exhibited no significant changes and were akin to the source genotypes. These findings suggest that the strategy of individually subjecting each polyploid to phenotypic screening for desired traits may not be an efficient and economical approach. The uncertainty in phenotypic outcomes highlights the immediate need to enhance our ability to control and refine genotype screening processes post-polyploidization.

With the advent of sequencing technologies, such as genomics, and the discovery of markers associated with genes/QTLs of interest, a more indirect selection and screening method called Marker-assisted selection (MAS) has emerged (Xu and Crouch, 2008; Ben-Ari and Lavi, 2012). MAS offers a promising solution for screening genotypes with desired traits after polyploidization, where specific molecular markers can be utilized to identify genotypes possessing the desired traits. Nonetheless, the presence of complexities, such as genome duplication, can present challenges when developing markers closely linked to the desired traits (Crossa et al., 2017). Moreover, MAS usually relies on a handful of loci with significant effects, which might fail to encompass the complete range of genetic variations accountable for the trait in question (Ben-Ari and Lavi, 2012; Olatoye et al., 2019). The influence of genome duplication

further complicates the situation. Therefore, accurately predicting the performance of a polyploid genotype based solely on its molecular markers can be a challenging task.

As compared to MAS, a more promising approach for polyploid screening generated from polyploidization could be GS, which uses genomic information to predict the performance of plants, enabling breeders to select desirable traits more efficiently and accurately (Newell and Jannink, 2014; Bhat et al., 2016). GS has been shown to be more effective than MAS in identifying desirable genotypes due to its enhanced accuracy, reduced reliance on specific markers, incorporation of non-additive effects, and reduced cost and time (Lorenz et al., 2011; Jonas and De Koning, 2013; Crossa et al., 2017). However, the predictive accuracy of the employed model is crucial for the effectiveness of GS, thus, careful selection and optimization of the prediction model are necessary to ensure its effectiveness.

9.3 Choosing the best individual: omics based genomic selection for polyploid screening

In general, GS includes all the genomics-driven strategies to select the best individuals from a testing population (TE) for breeding. The TE and the training populations (TR) are the key components of any genomic selection process. While TE refers to individuals with only genomic data, TR includes the group of individuals for whom both genomic and phenotypic data are available. In the current context, the individuals generated via polyploidization following genotyping will serve as the TE. Previously, traditional techniques such as PCR-based techniques and single nucleotide polymorphism (SNP) genotyping arrays have been used (Viana et al., 2016; Brault et al., 2021; Brault et al., 2022). However, in recent years, with the significant advancement and reduction in sequencing costs, crop breeders have shifted their focus towards omics-based strategies. The main advantage of multi-omics data in the genomic selection approach is its ability to enhance prediction accuracy by capturing diverse molecular interactions and factors influencing phenotypic traits (Ye et al., 2020; Sen et al., 2023). Although utilization of multi-omics data for GS in grapevine is lacking, transcriptome and metabolome data have been used for GS in maize breeding (Guo et al., 2016; Westhues et al., 2017).

Omics data such as genomics, transcriptomics, and metabolomics can provide valuable as well as novel insights on how to improve the precision of genomic relationship estimation in polyploids. Since the availability of the grapevine genome (initially in 2007 and later re-sequenced in 2019), omics-based studies have been extensively used to study polyploidy and

heterozygosity in grapevines (The French–Italian Public Consortium for Grapevine Genome Characterization, 2007; Liang et al., 2019). For example, in a recent study conducted by (Han et al., 2023), the authors used reference genome-based RNA-seq data analysis to identify the probable pathways involved in the freezing response in grapevines. Likewise, a few studies were conducted on grapevine breeding using various omics technologies (Wang et al., 2021; Savoi et al., 2022). In addition to genomics, metabolomics, and transcriptomics, derived and innovative omics (such as epigenomics and epitranscriptomics) can also be used for comprehensive understanding of the complex epigenetic modifications in the induced polyploids. Recently, there has been growing recognition of the important roles of epigenetic regulations and memories in the stress response of crops, including grapevines (Atanassov et al., 2022; Dal Santo et al., 2022; Jia et al., 2023). Epitranscriptomics, which deals with chemical modifications on RNA molecules, is yet to be applied in viticulture. However, there are some instances where epitranscriptomic study has been used for crop improvement (Hou and Wan, 2021). Nevertheless, despite the fact that these omics technologies can provide substantial insights into the molecular functioning of the genes of interest in grapes, they have several disadvantages, such as an inadequate view of biological processes. Crop traits and performance depend on multifaceted interactions between different biological components. Hence, the results obtained from single omics may miss the full system-level understanding required for effective understanding of the novel and influenced traits among the polyploid population. In this scenario, we recommend using more informative multi-omics data to get a comprehensive understanding of the grapevine traits and their genetic basis prior to TE selection. While multi-omics for breeding purposes grapevine are limited, these strategies are extensively used in other plant species such as rice (Sun et al., 2022), and maize (Farooqi et al., 2022). More details concerning the multi-omics in plant breeding can be found in Mahmood et al., 2022. In the context of grapevine breeding, data derived from multi-omics analyses can be used to identify the major genes that might enhance environmental adaptation and aid in the selection of crucial agronomic traits. Inferring the exact link between the genes and the final phenotype might be difficult due to the lack of middle omics (from genomics to phenomics). Integration of genome-wide association studies (GWAS) with other omics (such as metabolomics and transcriptomics) will reduce the variety of candidate genes and aid system analysis of gene function. For instance, GWAS integration with transcriptome-wide association studies (TWAS) can be used to discover expression QTLs (eQTLs) (fine-mapping technique) in the induced polyploid grapevines. This approach can be an excellent option to establish the relationship between transcript abundance and phenotypic variance while simultaneously gaining insights into the

regulatory functions of genetic variations responsible for phenotypic changes. Earlier, the GWAS-TWAS integrative approach was used in rice (Anacleto et al., 2019; Mahmood et al., 2022) and cotton (Li et al., 2020; Mahmood et al., 2022). Combined GWAS and metabolome-wide association studies (MWAS) can simultaneously screen a vast number of grapevine accessions for possible associations between their genomes and diverse metabolites. This collaborative approach will offer significant insights into the genetic basis of complex traits and the level of metabolic diversity within the population. Furthermore, the integration of the eQTLs and metabolite quantitative trait loci (mQTLs) can also complement GWAS while predicting the phenotypic outcomes of the induced polyploid genotypes. This integration contemplates the variations in mRNA expression and metabolite production and will provide novel insights into the eventual performance of the produced varieties in a comparatively short time period, which otherwise would be time-consuming. The multi-omics datasets can be integrated via correlation-based integration, network-based integration, and pathway-based integration. In the context of multi-omics data integration, correlation-based methods aim to identify patterns of co-expression across different omics datasets, whereas network-based integration focuses on creating biological networks representing various interactions between biomolecules, followed by the integration of omics data onto these networks. The pathway-based integration method focuses on mapping the omics data onto predefined biological pathways. Even though these methods have their own advantages and disadvantages, in practice, the choice of the appropriate method depends on the research question and the availability of data. Often, a combination of these methods can be used for a more comprehensive understanding at the organismal level. More details on systematic multi-omics data integration approaches can be found in Fabres et al., 2017 and Jamil et al., 2020.

The genotyping of the TR population is followed by its phenotyping. To ensure accurate phenotyping, it's important to carefully design experiments and select appropriate traits to measure. Selection of traits and prioritization should be relevant to the goals of crop improvement, such as yield, disease resistance, drought tolerance, or nutritional quality. The previous GS studies on grapevines assessed various traits related to agronomical characteristics, drought tolerance, and yield components. These studies aimed to enhance understanding of these traits' genetic architecture and identify molecular markers associated with their variation (Viana et al., 2016). Although data complexity increases, including more traits might provide a broader representation of phenotypic variation, allowing for a more comprehensive assessment of an individual's genetic potential. For instance, (Flutre et al., 2022) phenotyped 279 *Vitis*

vinifera training cultivars and assessed a total of 127 traits. Additionally, they also combined several other traits, making a total of 152. Despite using an extensive dataset, the study achieved high prediction accuracy for 50% of the response variables. Once phenotypic and genotypic data have been obtained from the TR population, they can be employed to construct prediction models, using phenotype as the response and genotype as the predictor. To date, several parametric models such as genomic best linear unbiased prediction (GBLUP), Bayesian regression-based methods (like BayesA), sparse linear mixed model methods (like BayesB), and Bayesian least absolute shrinkage and selection operator (BLASSO) methods (like BayesC) have been developed for GS. These models address different challenges and offer unique advantages. For a comprehensive understanding of these statistical models, one can refer to Budhlakoti et al. (2022). Previous GS studies in grapevines compared different prediction models and evaluated their performance. For example, Flutre et al. (2022) compared two multi-SNP models and determined that the dense RRBLUP/GBLUP model was a relevant default, while the sparse varbvs model achieved higher accuracy for traits closer to genetic variation. Brault et al. (2022) used Ridge Regression (RR) and LASSO models and found that predictive ability varied depending on the scenario and trait. In the current context, BLASSO could be an appropriate option as it provides a probabilistic framework that can accommodate uncertainty in variable selection, making it useful when dealing with multiple omics layers where interactions may be complex. Although it is important to note that each model has its strengths and weaknesses, the selection of a suitable model depends on specific objectives, genetic architecture, and available data. Comparisons and evaluations of different models are often recommended for optimal performance in GS. Following the model's development, the next step is to select and validate the model. After a prediction model has been prepared and validated, it can be used to predict the Genomic Estimated Breeding Values (GEBVs) of individuals in the breeding population. The GEBVs can then be used as a parameter to rank individuals in the breeding population according to their predicted genetic merit for the trait of interest. Figure 9.1 describes the potential screening of elite genotypes through omics-integrated genomic selection in a polyploid population generated via *in vitro* polyploidization.

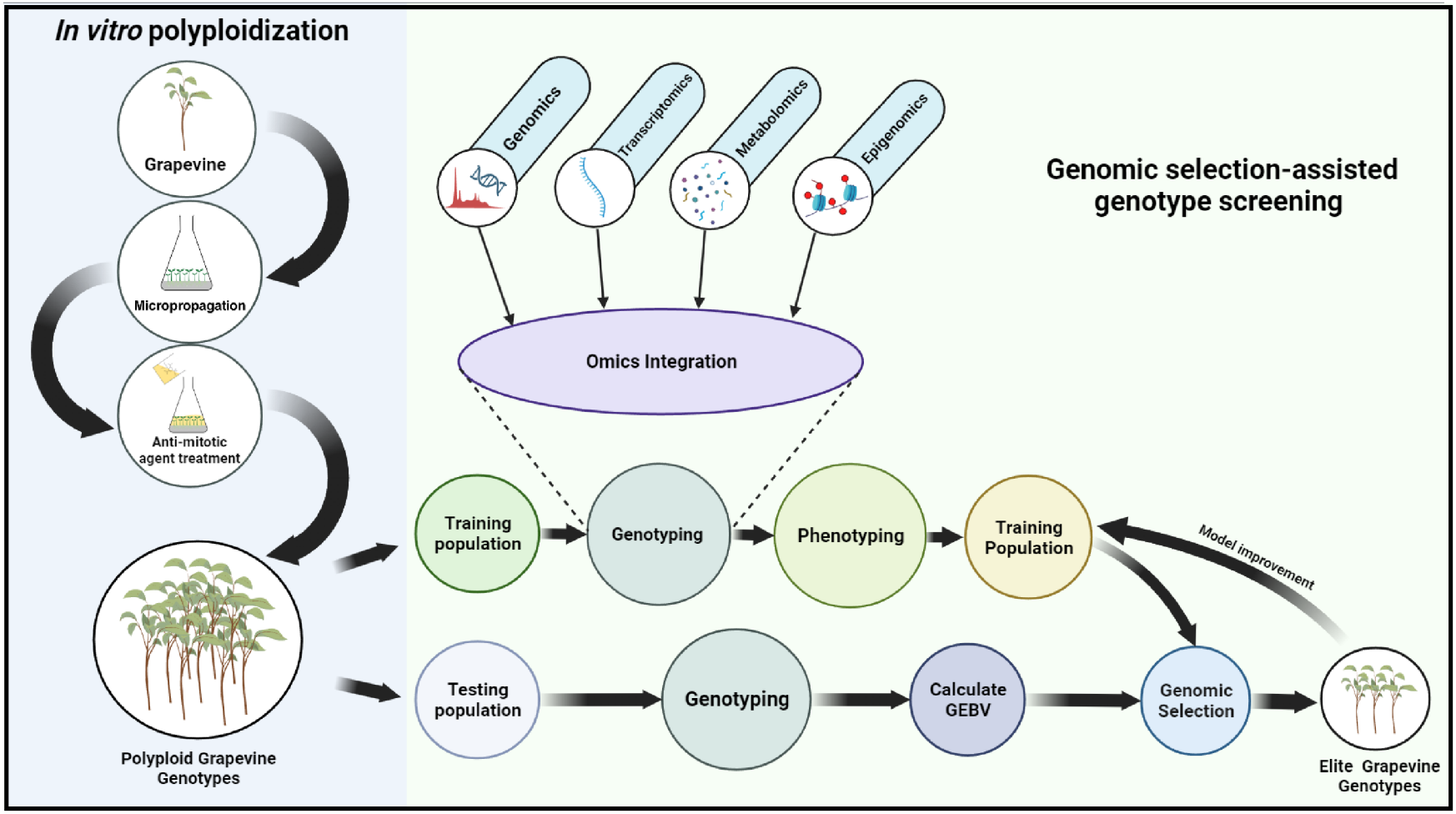

Figure 9.1 Flow diagram illustrating potential elite genotype screening via omics integrated genomic selection among polyploid population generated through *in vitro* polyploidization. The diagram depicts the various phases involved in genomic selection. By utilizing genotypic and phenotypic data acquired from the training population, the genomic selection models can be optimized, enabling the estimation of breeding values for superior genotypes.

9.4 Current challenges and the way forward

Artificial polyploidization in grapevines presents a bottleneck in plant breeding, but the use of GS for genotype screening following polyploidization offers a promising approach to address this issue. Although, for successful prediction of elite polyploid genotypes, it is crucial to consider the potential shortcomings to avoid or address them. Genotyping the produced grape polyploids via omics-based GS can introduce unique challenges, such as integration of the multi-omics data along with their proper management and interpretation and functional annotation and biological relevance of omics markers. Multi-omics datasets, which are often produced using diverse technologies and platforms, lead to data heterogeneity, besides producing a high-dimensional and complex data landscape. Hence, despite being informative and precise, the volume and complexity of the data make its management difficult, which might require dedicated tools and algorithms which are competent of handling the dimensionality and complexity of multi-omics data. In addition to these, multi-omics datasets usually contain a large number of variables and features, which complicates the downstream analyses. Besides data complexity, we also recommend focusing on reference genome availability and trait-marker associations. Alongside reference genome availability, detection of the biologically significant links between omics markers and complex traits in polyploids can also be intricate.

Synthetic polyploids are known to have better adaptability to a wide range of environments compared to their diploid counterparts. However, it is important to assess the Genotype × Environment (G×E) interactions, as they can greatly influence the predictive potential of GS (Mulder, 2016; Jarquín et al., 2017). Additionally, G×E is particularly relevant in crops such as grapevines, which are highly sensitive to environmental factors that could influence both the quantitative and qualitative characteristics of the crop (Dinu et al., 2021; Dal Santo et al., 2022). Another potential challenge is that, while traditional models show success in prediction, they often overlook vital non-additive effects like genomic imprinting and epistasis, impacting prediction accuracy (Jackson and Chen, 2010; Endelman et al., 2018; Varona et al., 2018; Hunt et al., 2020). Artificially induced polyploids exhibit substantial non-additive effects on phenotype, particularly notable in grapevines propagated by cutting and grafting, influencing traits and stress responses (Tan et al., 2023). Understanding and utilizing these additive effects is crucial for effective genomic prediction in grapevine breeding. For that, machine learning models like random forests, support vector machines, and deep neural networks could be instrumental due to their ability to capture complex marker-trait relationships, select markers, and handle noise (Heslot et al., 2014; Crossa et al., 2017; Wang et al., 2018; Van Dijk et al.,

2021). Despite several challenges, integrated polyploidization and GS strategy could be an excellent option for grapevine breeding. An updated and detailed understanding of the associated challenges will be the main key. Active collaboration between the experts in genomics, bioinformatics, statistical genetics, and grape breeding along with innovations in technology as well as data analysis methods will definitely enable us to overcome these impediments and leverage the full potential of omics-based GS in grape polyploids.

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10. Conclusions

In vitro polyploidization or artificial chromosome doubling represents an underutilized breeding approach with substantial potential for enhancing the genetic profile of medicinal plants. The resulting polyploid plants are often known for their remarkable improvements of various traits, which can be advantageous across multiple applications, including medicinal properties. In the case of commercially important plants (such as plants with medicinal properties), this augmented growth can significantly increase the biomass and lead to significant economic gains. Apart from the growth benefits, *in vitro* polyploidization has also been known for its ability to stimulate the production of secondary metabolites. Hence, the current thesis focused on the potential of this innovative plant-breeding technique to enhance the agronomical and medicinal properties of plants. The results presented in this thesis carry profound implications for the field of medicinal plant genetics and breeding.

The thesis successfully induced polyploidy using *in vitro* polyploidization in the studied plants, including spearmint, lemon balm, basket plant, and mini melon. Oryzalin as an anti-mitotic agent was found to be effective in inducing polyploidization in all the studied plant species. The highest polyploid induction rate in all plant species ranged between 8% to 15%. Additionally, the thesis assessed the effect of artificial polyploidization on various morphological, biochemical, biological, photosynthetic, and molecular characteristics.

The first study aimed to assess the efficacy of oryzalin as an anti-mitotic agent in inducing polyploidy in *M. spicata* and produce polyploid plants with enhanced morphology and essential oil levels. Spearmint is a perennial herb renowned globally for its wide range of ethnopharmacological properties. Despite its significance, no studies (to the best of our knowledge) have explored synthetic polyploidization in this species for enhancing essential oil yield. In our study, we successfully optimized the oryzalin-based polyploid induction method in M. spicata. Despite obtaining a relatively low induction frequency, we produced genotypes with superior agronomical traits suitable for commercial demands. Interestingly, in one of the hexaploid spearmints, a 48.85% increase in essential oil yield (with significant boosts in carvone and limonene) was observed compared to the control.

The second study involved the induction of polyploidy in *M. officinalis*. The induced tetraploid plants displayed vigorous growth, featuring longer shoots, larger leaves, and a higher leaf count. The induced tetraploid population exhibited higher photosynthetic performance and larger peltate trichomes, which eventually led to a 75% increase in average essential oil yield. The

analysis of essential oil composition in both diploid and tetraploid plants revealed three main components: geranial, neral, and citronellal. While the level of citronellal remained unchanged, the geranial and neral levels increased in polyploid plants. Geranial and neral are highly valued for their antioxidant, antimicrobial, and anti-inflammatory properties. Additionally, these compounds also aid in digestion and relieve digestive discomforts.

In the third study, we conducted a comprehensive morphological, cytological, and molecular comparison between diploid and induced autotetraploid basket plant (*C. fragrans*). The induced polyploid Basket plant displayed distinct morphological features, including significantly increased leaf and flower sizes compared to diploid plants. Elemental analysis revealed higher sodium, iron, and calcium content in tetraploid plants, along with a 100% increase in potassium content.

Mini melon fruits also exhibited significant morphological variability in the fourth study. Cultivation and evaluation of polyploid and control diploid genotypes under greenhouse and field conditions revealed substantial morphological differences in flowers, fruits, and leaves. Nutritional analysis demonstrated improvements in key parameters such as glucose, fructose, carotenoid, polyphenol, and antioxidant contents in the fruits of induced polyploids compared to diploid plants. This finding is particularly noteworthy as heightened sugar content can augment the sweetness and overall appeal of fruits to consumers. Such insights into fruit composition variations shed light on the potential for improving fruit quality through *in vitro* polyploidization technique.

The focus shifted to grapevines in the fifth study, where the thesis systematically identified the current state of polyploidization in *Vitis* genus. *In vitro* polyploidization was found to be more effective and suitable for inducing polyploidy in *Vitis* spp. compared to the *in vivo* approach. Despite having huge potentials, there are several bottlenecks associated with *in vitro* polyploidization, such as genomic instability and reduced fertility. Although, the most important challenge is to select the most promising polyploid varieties. In such a scenario, GS and multi-omics approaches might offer a promising possibility for predicting the most economically desirable genotypes. By exploring the genomic profiles of produced polyploids, GS can identify and select individuals with the most desirable traits, such as increased yield, enhanced quality, or better resistance to diseases and pests.

In conclusion, these research outcomes open avenues for utilizing *in vitro* polyploidization as a strategic tool in the genetic improvement of medicinal plants. The findings highlight the efficacy of oryzalin as an antimitotic agent and its role in inducing polyploidy in the studied plants. The enhanced traits observed in the polyploid genotypes underscore the potential of *in vitro* polyploidization for developing improved genotypes with heightened agronomical and medicinal properties. This has direct implications for industries reliant on these plants for pharmaceutical, culinary, or ornamental purposes. The findings of this thesis not only confirm the feasibility of *in vitro* polyploidization for genetic enhancement but also pave the way for targeted and species-specific applications. Furthermore, while acknowledging the challenges associated with *in vitro* polyploidization, such as genomic instability and reduced fertility, this research provides valuable insights into addressing these challenges and optimizing the breeding process. As the demand for bioactive compounds from medicinal plants continues to rise, the insights gained from this research significantly contribute to ongoing efforts to optimize cultivation practices and secure a sustainable supply of high-quality medicinal plant products.

10.1 Recommendations for future work

While this thesis has made significant strides in exploring the potential of *in vitro* polyploidization for the genetic improvement of medicinal plants, several avenues for future research emerge. These recommendations aim to build upon the current findings and address certain aspects that warrant further investigation.

Complementing morphological and physiological assessments, future research could employ transcriptomic and metabolomic analyses to elucidate the molecular mechanisms triggered by polyploidization. Understanding changes at the molecular level will contribute to a more comprehensive understanding of the observed improvements in biochemical and physiological parameters.

While improvements in morphological traits have been observed, future studies should focus on validating the enhanced medicinal properties of polyploid variants. This involves rigorous analyses of bioactive compound concentrations, pharmacological assays, and clinical studies to ascertain the therapeutic efficacy of polyploid-derived medicinal plants. Although biological activities of the fruits obtained from the induced polyploids of mini melon were carried out, biological activities improvement in the other polyploids (spearmint, lemon balm, basket plant) obtained in the current thesis needs to be part of future studies.

An essential aspect for future investigations involves assessing the generated polyploids for enhanced resilience to both abiotic and biotic stresses. Understanding how polyploidization influences responses to environmental challenges, such as drought, salinity, and pathogen attacks, will provide valuable insights into the adaptability and robustness of these novel genotypes.

In conclusion, these recommendations offer a roadmap for future research endeavors in the realm of *in vitro* polyploidization for medicinal plant improvement. Addressing these aspects will not only refine the understanding of the underlying mechanisms but also enhance the practical applications of this innovative approach in the broader context of medicinal plant genetics and breeding.

11. References (not listed in results)

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Zahumenická, P., Fernández, E., Šedivá, J., Žiarovská, J., Ros-Santaella, J. L., Martínez-Fernández, D., et al. (2018). Morphological, physiological and genomic comparisons between diploids and induced tetraploids in *Anemone sylvestris* L. *Plant Cell, Tissue and Organ Culture* 132, 317–327. doi: 10.1007/s11240-017-1331-3

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12. Appendices

12.1 Curriculum vitae

Rohit Bharati

Nationality: Indian | **Date of birth**: 12/01/1997

Phone number: (+420) 774963883 | **Languages**: English, Hindi, Bengali

Email address: rb79750@gmail.com

Current Address: Kamýcká 1280, 16521 Prague, Czech Republic

EDUCATION

Ph.D. in Agricultural Specialization [02/12/2019 – Current] [Expected Thesis Defense by April 2024], *Czech University of Life Sciences, Prague, Czechia*
Thesis: Exploring the Potential of *In-vitro* Polyploidization for Genetic Improvement of Medicinal Plants

Master of Science in Agricultural Biotechnology [21/07/2017 – 04/07/2019]
Ramakrishna Mission Vivekananda Educational and Research Institute, Kolkata, India
Thesis: Comparative study of the growth behavior of in-vivo and in-vitro plants of *Withania somnifera* with their antifungal activity.

Bachelor of Science in Biotechnology [20/07/2014 – 06/2017]
Maulana Abul Kalam Azad University of Technology, West Bengal, India

PROFESSIONAL EXPERIENCE

Scientific laboratory technician

Laboratory of Plant Tissue Cultures, Czech University of Life Sciences Prague, Czechia [01/01/2021 – Current]
Roles:
Media Preparation | Glassware and Equipment Sterilization | Surface Sterilization, Micropropagation, and Subculturing | Inventory Management | Helping and Guiding Bachelor and Master Students | Maintaining Safety Measures

Research Assistant

Crop Research Institute, Prague [31/07/2020 – 31/10/2021]
Research Area: Plant-Virus Interaction (Worked with Triticum, Brassica, and Nicotiana spp.)
Roles:
Experiment Designing| Homogenize Plant samples | Extracting RNA & DNA from samples| cDNA synthesis | real-time PCR|

PEDAGOGICAL ACTIVITY

» **Undergraduate Lecturer |** *Faculty of Tropical AgriSciences, Czech University of Life Sciences, Prague, CZ*

Subject: Tropical Agricultural Systems (IRI007E)

- Batch 2022/2023 (40 students)
- Batch 2021/2022 (29 students)
- Batch 2020/2021 (25 students)

» **Graduate student mentor |** *Laboratory of Plant Tissue Cultures, FTA, CZU*

- Mentored four students: 3 Bachelor's and 1 Master's student, providing guidance in their experiments and research projects in Plant tissue culture and biotechnology.

TRAINING AND INTERNSHIP EXPERIENCE

» **Internship** at *Slovak University of Agriculture, Nitra, Slovakia*
[01/11/2021 – 31/12/2021]

Throughout my internship, I conducted an investigation into the impact of ploidy levels on medicinal herbs belonging to the Lamiaceae family. This study involved the utilization of advanced instrumentation dedicated to the assessment of plant photosynthesis, including PhotosynQ, SPAD, Multiplex, CIRAS, and Dual PAM. Additionally, I refined my proficiency in data analysis, ensuring precise interpretation and the derivation of insightful conclusions from the acquired datasets.

» **Summer Training** at *Universitat Politècnica de València, Valencia, Spain*
[13/07/2021 – 22/07/2021]
Topic: *In vitro* Techniques and Research of Mediterranean Plant Biodiversity

PROJECTS PARTICIPATED

- Genetic improvement of medicinal plants through biotechnological interventions - (IGA FTZ-20233105) [04/2022 – 12/2023]
  Role: Co-investigator
- Development of polyploid genotypes in Mentha spicata using in vitro somatic polyploidization - (IGA FTZ-20223101) [01/2021 – 12/2022]
  Role: Co-investigator
- In vitro induced polyploidy in *Thymus camphoratus* Hoffmanns (IGA FTZ 20213113) - (IGA FTZ-20213105) [04/2020 – 10/2021]
  Role: Co-investigator
- In vitro indukovaná polyploidie u Celosia argenta L. (IGA FTZ-20205004) [04/2019 – 12/2020]
  Role: Co-investigator

Publication related to the thesis

Bharati, R., Fernández-Cusimamani, E., Gupta, A., Novy, P., Moses, O., Severová, L., ... & Šrédl, K. (2023). Oryzalin induces polyploids with superior morphology and increased levels of essential oil production in *Mentha spicata* L. *Industrial Crops and Products*, *198*, 116683. **[IF: 6.44]**

Bharati, R., Gupta, A., Novy, P., Severová, L., Šrédl, K., Žiarovská, J., & Fernández-Cusimamani, E. (2023). Synthetic polyploid induction influences morphological, physiological, and photosynthetic characteristics in *Melissa officinalis* L. *Frontiers in Plant Science*, 14., **[IF: 5.6]**

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. **[IF: 3.94]**

Fernández-Cusimamani, E., **Bharati, R.**, Javůrková, T. A., Škvorová, P., Paznocht, L., Kotikova, Z., ... & Orsák, M. (2023). Artificial Polyploidization Enhances Morphological, Physiological, and Biological Characteristics *in Melothria scabra* Naudin. Horticulturae, 10(1), 22. **[IF: 3.1]**

Bharati, R., Sen, M. K., Severová, L., Svoboda, R., & Fernández-Cusimamani, E. (2023). Polyploidization and Genomic Selection Integration for Grapevine Breeding: A Perspective. *Frontiers in Plant Science*. **[IF: 5.6]**

Other Publications

Bharati, R., Sen, M. K., Kumar, R., Gupta, A., Žiarovská, J., Fernández-Cusimamani, E., & Leuner, O. (2023). Systematic Identification of Suitable Reference Genes for Quantitative Real-Time PCR Analysis in *Melissa officinalis* L. *Plants*, *12*(3), 470. **[IF: 4.65]**

Bharati, R., Sen, M. K., Kumar, R., Gupta, A., Sur, V. P., Melnikovová, I., & Fernández-Cusimamani, E. (2022). Selection and validation of the most suitable reference genes for quantitative real-time PCR normalization in *Salvia rosmarinus* under in vitro conditions. *Plants*, *11*(21), 2878**. [IF: 4.65]**

Sen, M. K., Bhattacharya, S., **Bharati, R.**, Hamouzova, K., & Soukup, J. Comprehensive Insights into Herbicide Resistance Mechanisms in Weeds: A Synergistic Integration of Transcriptomic and Metabolomic Analyses. *Frontiers in Plant Science*, 14, 1280118. **[IF: 5.6]**

Hamouzova, K., Sen, M. K., **Bharati, R.**, Košnarová, P., Chawdhery, M. R. A., Roy, A., & Soukup, J. (2023). Calcium signalling in weeds under herbicide stress: An outlook. *Frontiers in Plant Science*, *14*, 1135845. **[IF: 6.62]**

Sen, M. K., Hamouzová, K., Mikulka, J., **Bharati, R.,** Košnarová, P., Hamouz, P., ... & Soukup, J. (2021). Enhanced metabolism and target gene overexpression confer resistance against acetolactate synthase-inhibiting herbicides in *Bromus sterilis*. *Pest Management Science*, *77*(4), 2122-2128. **[IF: 4.84]**

Žiarovská, J., Urbanová, L., Montero-Torres, J., Kováčik, A., Klongová, L., **Bharati, R.,** ... & Leuner, O. (2023). Polymorphism of Bolivian accessions of *Arachis hypogaea* L. revealed by allergen coding DNA markers. *Plant, Soil and Environment*, 69(12), 615-627. **[IF: 2.4]**

CONFERENCES AND SEMINARS

The 3rd International Electronic Conference on Agronomy

Enhancing Essential Oil Yield and Agronomical Traits in *Melissa officinalis* L. through Synthetic Polyploidization [Virtual Conference, 15/10/2022 – 30/10/2022] Link: https://sciforum.net/paper/view/14966

Tropentag 2022

Development of polyploid genotypes in *Mentha spicata* using in vitro somatic polyploidization [Czech University of Life Sciences Prague, Czech Republic, 14/09/2022 – 16/09/2022] Link: http://dx.doi.org/10.13140/RG.2.2.22828.95361

Tropentag 2021

Genetic and morphological stability of autopolyploid *Thymus vulgaris* L. and changes in its anatomy and physiology [University of Hohenheim, Germany, 15/09/2021 – 17/09/2021]

ELLS Scientific Student Conference 2020

Effect of zinc oxide and titanium dioxide nanoparticles on in vitro micropropagation of *Monarda didyma* L. from nodal segments [University of Natural Resources and Life Sciences, Vienna, Austria] [19/11/2020 – 21/11/2020]

REVIEW ACTIVITY

Participated as an academic reviewer for the following prestigious journals :

- Journal of Plant Growth Regulation (Q1)
- Scientia Horticulturae (Q1)
- Pest Management Science (Q1)

AWARDS AND RECOGNITION

Josef Hlávka Award for Outstanding Ph.D. Achievement [October 2023]

The prize is intended for talented students up to the age of 33 who have shown special ability and intellectual creativity in their branch.

Rector's Award for Excellence publication output 2023 [November 2023]

Recognized with the Rector's Award for delivering an exemplary publication as a Ph.D. student at the Czech University of Life Sciences, highlighting a significant contribution to research.