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Morphological and molecular comparison between diploids and induced autotetraploid plants in *Callisia fragrans* Lindl. (Woodson).

MASTER'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled Morphological and molecular comparison between diploids and induced autotetraploid plants in *Callisia fragrans* Lindl. (Woodson) independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague 20. 4. 2022

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Kateřina Beranová

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Abstract

The objective of the current study was to determine morphological and molecular differences between diploids and induced autotetraploid plants in *Callisia fragrans* Lindl. (Woodson). Diploid plants 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 analyzed using flow cytometry and chromosome counting. Among all treatments, six tetraploid plants (2n = 4x = 24) were obtained after 8 weeks in MS media containing 5 μM oryzalin. Upon successful establishment in the greenhouse, tetraploid plants were morphologically distinct compared to the diploid plants. The size of the leaf increased significantly, and flower size nearly doubled when compared to the mother diploid plant. Inductively coupled plasma atomic emission spectroscopy was further utilized to compare the macro and micronutrients between diploid and tetraploid plants. Tetraploid plants exhibited significantly higher content of sodium, iron, and calcium. Interestingly, potassium content in the tetraploid was doubled. Molecular analysis utilizing iPBS and CDDP markers exhibited completely polymorphic profiles depicting variation between tetraploid and diploid genotypes. In *C. fragrans*, these molecular analyses were applied for the first time. In vitro polyploidization using oryzalin could effectively be used in inducing polyploids in this specie. Additionally, the results obtained in this study will provide a basis for future breeding opportunities in this species.

Key words: Flow-cytometry, Nutrient content, Medicinal plant, Oryzalin, Commelinaceae, Molecular analysis

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List of the abbreviations used in the thesis

MS medium	Media Murashige & Skoog (1962)
BAP	6-Benzylaminopurine
NAA	1-Naphthaleneacetic acid
Ribavirin	1-(β-D-Ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide
Trifluralin	2,6-dinitro-N, N-dipropyl-4-(trifluoromethyl) benzenamin
Oryzalin	3,5-dinitro-N4, N-4-dipropylsulfanilamid
HPLC	High-performance liquid chromatography
EMS	Ethyl methanesulfonate
RFLP	Restriction fragment length polymorphism
PCR	Polymerase chain reaction
iPBS	inter-PBS amplification
RAPD	Random amplified polymorphic DNA
AFLP	Amplified fragment length polymorphism
SSR	Simple sequence repeats
SNP	Single nucleotide polymorphism
LTR	Long terminal repeats
PBS	Primer binding site
CDDP	Conserved DNA-Derived polymorphism
p.o	per os
FRAP	Ferric Reducing Antioxidant Power

1. Introduction and Literature Review

1.1. Introduction

Basket plant (*Callisia fragrans* (Lindl.) Woodson: 2n=2x=12) is a wild, perennial, medicinal plant belonging to a Commelinaceae family along with other 500 species. This monocotyledonous weedy growing plant originally from Mexico, is now globally distributed (Chernenko et al. 2007; Malakyan et al. 2015; Saensouk & Saensouk 2020). It is known to be a homegrown modesty plant with medicinal usage. In folk medicine it is used for the treatment of joint disorders, inflammation, burns, wounds and similar diseases (Malakyan et al. 2015; Pranskuniene et al. 2019). Chernenko et al. (2007) analyzed that *C. fragrans* contains various bioactive components, such as phenolic compounds, amino acids, carbohydrates, flavonoids, coumarins, vitamins etc. These compounds are responsible for treatment illnesses such as oncological diseases, cardiovascular problems, tuberculosis, asthma, gastrointestinal disorders, healing of burns and wounds (Chernenko et al. 2007; Malakyan et al. 2015; Karapetyan & Mairapetyan 2020; Phan et al. 2020).

The breeding of medicinal plants focuses on obtaining new varieties with higher content of secondary metabolites. Numerous breeding methods are used, namely hybridization, heterogenic breeding, mutagenesis, polyploidy breeding and genetic engineering. One of the most common methods is *in vitro* somatic polyploidy. Although it is an older method, it is still effective in breeding new genotypes. This is based on multiplication of somatic chromosomes induced by using antimitotic agents (Dehghan et al. 2017; Eng & Ho 2019; Niazian & Nalousi 2020). The most applied antimitotic agents are colchicine (Talebi et al. 2017; Esmaeili et al. 2020) and oryzalin (Parsons et al. 2019; Shmeit et al. 2020). Chromosome doubling often causes useful changes such as improving biomass, amount of bioactive compounds and environmental conditions (stress) adaptability (Dhooghe et al. 2011; Dehghan et al. 2017; Sabzehzari et al. 2019).

For example, polyploidization increased essential oil content in *Thymus vulgaris* (Shmeit et al. 2020) and *Chamaemelum nobile* (Tsuro et al. 2016). Higher phenolic acids

content (cichoric, caffeic, chlorogenic, caftaric, and 1,5-dicaffeoyl quinic acid) was detected in tetraploid *Echinacea purpurea* (Mei et al. 2020), total phenolic and flavonoid content was increased by polyploidy induction in *Salvia officinalis* (Hassanzadeh et al. 2020). Similar results were obtained for carotenoid content in *Physsalis alkekengi* (Santos et al. 2020) and *Zingiber officinale* (Zhou et al. 2020).

According to our knowledge, successful *in vitro* induced somatic polyploidization in *Callisia fragrans* has not yet been achieved. So far, only experiments with *in vivo* applied colchicine to roots have been published (Roy 1973; Singh & Roy 1990). This study determines chemical, morphological and molecular variation in newly obtained autopolyploid plants compared to diploids plants (2n=2x=12).

1.2. Taxonomy and botanical description

Order Commelinales contains monocot, mostly tropical plants. About 920 species are included in 60 genera which are organised in 5 families. Order Commelinales content families: Commelinaceae, Haemodoraceae, Hanguanaceae, Pontederiaceae, Philydraceae a (Chernenko et al. 2007; Zuntini et al. 2021).

This order contains lot of species, which are cultivated for many qualities. Some species are cultivated for its ornamental purposes for example spiderworts (*Tradescantia* spp.) and kangaroo paws (*Anigozanthos* spp.). Haemodoraceae are reported as a good source of pigments. *Haemodorum* spp, called a bloodroot, contains arylphenalenones in their stems and roots and produce a reddish colour for brown and purple dyes. Some of this species have also edible roots. *Haemodorum corymbosum*, which is known as a colourant, has additionally antibacterial and antitumor properties (Anderson & Sax 1936; Zuntini et al. 2021).

1.2.1. Family Commelinaceae

Commelinaceae is called as the spiderwort or dayflower family and is the biggest family in order Commelinales (Saensouk & Saensouk 2020). It consists of tropical and subtropical perennial herbs, which are spread widely in those climatic zones (Arroyo et al. 2001; Saensouk & Saensouk 2022). Plants belonging into this family could be recognised by succulent stems and sheathing leaves. Family Commelinaceae includes approximately 39 existing genera including about 749 species (Taylor 2009, Zuntini et al. 2021). Most of the species belongs in to two main subfamilies: Commelineae which includes 13 genera, for example *Commelina, Murdannia* and *Aneilema* and subfamily Tradescantieae which contains 25 genera, for example *Callisia, Tradescatiea* and *Gibasis* (Anderson & Sax 1936; Zuntini et al. 2021; Saensouk & Saensouk 2022).

A lot of species in this family are grown for its ornamental purposes and for ethnobotany (Chumroenphat & Saensouk 2021). Due to a vigorous growth of most of Commelinaceae species, lots of them are considered as a weed (*Commelina obliqua, C. maculata, Murdannia vaginata, M. spirata, Tradescantia zebrina, T. pallida, T. spathacea* and *Cyanotis cristata*). *Murdannia nudiflora* (Figure 1) is frequently occurred in cocoa, sugarcane, coffee, maize and banana fields (Das et al. 2021). *Commelina benghalensis* is major weed in India in groundnut, rice, soya bean and cotton fields. Despite this *C. benghalensis* (Figure 2) is an important medicinal plant in folk medicine. In Pakistan it is used for the treatment of swellings, leprosy. In India and China, it is used as an anti-inflammatory, laxative, diuretic and depressant (Shaikh et al. 2021). *C. benghalensis* has a potential anti-cancer effect (Batool et al. 2020). Even though *T. zebrina* (Figure 3) and *T. pallida* (Figure 4) are vigorous weeds, are also cultivated for its ornamental purple leaves. *T. pallida* is also used as a medicinal plant in Thailand (Chumroenphat & Saensouk 2021). *Tradescantia fluminensis* var. foliis variegatis which is native to Brazil is grown for ornamental purposes due to its striped sheathed leaves. Some species are edible, for example *Commelina cyanea* and *C. benghalensis* have edible leaves, which are used as a vegetable (Hunt 1986; López-ferrari et al. 2014; Chumroenphat & Saensouk 2021; Zuntini et al. 2021).

Figure 1. *Murdannia nudiflora*

Source: Glorioso, 2021

Figure 2. *Commelina benghalensis*

Source: Hues, 2021

Figure 3. *Tradescantia zebrina*

Source: Trilling, 2022

Figure 4. *Tradescantia pallida*

Source: Ramirez, 2021

1.2.2. Genus *Callisia* Loefl.

This genus includes about 25 species, where most of them originates in Central America and some in area of Southwest USA and other parts of tropical America. Nowadays are pantropical spreaded (Figure 5; Grabiele et al. 2015). Genus *Callisia* is different by number of chromosomes and its morphology. In various research is published about 15 species with cytotypes and karyotypes ranges from 2n = 12 up to 2n = 72 (Faden 1998). Genus *Callisia* involves 6 sections, which are divided by common haploid chromosome number (Table 1). *Callisia fragrans* (2n = 12) belongs into the section "Callisia", corporately with *C. repens* (2n = 12), *C. gentlei* (2n = 12), *C. soconuscensis* (2n = 12). Most of this species originates in Mexico. *C. repens* and *C. monandra* have its origin in Argentina (Grabiele et al. 2015; Saensouk & Saensouk 2020).

Section "Callisia" includes succulent or sub succulent perennial plants which could be recognized by spirally arranged leaves which are formed in bromeliiform rosettes or two-ranked, ovate to oblong-lanceolate shaped. Roots are fibrous. Flowers are formed in a spike-like or paniculate inflorescences. Sepals are pale, petals are pink or white with or without expanded blade. Mostly 6 stamens occur, sometimes 3-1. All stamens are fertile, infrequently 1 or more are staminodial (Hunt 1986; Nikolaeva & Nikolaeva 2009; Malakyan et al. 2015).

Callisia species are cultivated for many properties. Some species have attractive morphology. Dominantly the beautiful various coloured species are cultivated as ornamentals and medicinal plants, such as creeping inch plant (*C. repens*) and dragon tail (*C. soconuscensis*). *C. repens* is frequently used in Indian gardens, where it is cultivated for its ornamental leaves and medicinal uses (Hunt 1986; Nandikar et al. 2010; Saensouk & Saensouk 2020). It is known that *C. repens* is lowering the blood sugar and its leaves are also edible (Leng et al. 2019; Chumroenphat & Saensouk 2021).

Table 1. Division of sections in genus *Callisia* Loefl. (Anderson & Sax 1936; Hunt 1986; Molgo 2017)

Section	Common haploid chromosome number	Species examples	Origin
Hadrodemas	n = 8	*C. warsezewicziana*	North and South America, Karibic islands
Cuthbertia	n = 6	*C. graminea; C. ornata; C. rosea*	Endemic in the USA
Lauia	n = 6	*C. lauia*	Endemic in the Mexico
Brachyphylla	n = 6, 8	*C. navicularis*	USA and Mexico
Leptocallisia	n = 7	*C. monarda*	USA
Callisia	n = 6	*C. fragrans; C. repens, C. gentlei,*	Mexico and S. America

Figure 5. Distribution of genus *Callisia* Loefl. in the world

Source: "Discover life" 2022

1.2.3. *Callisia fragrans* (Lindl.) Woodson

Callisia fragrans (Lindl.) Woodson is perennial herb, wildly growing in Mexico, where originates (Hunt 1986; Malakyan et al. 2015). Due to a special design of inflorescence, the name basket plant is used. In lot of countries, it is grown as an ornamental indoor plant for about 100 years (Nikolaeva & Nikolaeva 2009; Malakyan et al. 2015).

Taxonomy of *Callisia fragrans* (Lindl.) Woodson:

- Kingdom Plantae
- Phylum Tracheophyta
- Class Liliopsida
- Order Commelinales
- Family Commelinaceae
- Genus *Callisia* Loef.
- Species *Callisia fragrans* (Lindl.) Woodson

Source: The Plant List 2013, GBIF 2022

C. fragrans origin is firstly reported by Darlington (1929). Origin is assessed in SE Mexico (Hunt 1986; Malakyan et al. 2015). Currently it is found in all tropical parts of Central and South America and is widespread in the Pacific Islands. It also widely spread to Australia (Figure 6; Heywood 1993).

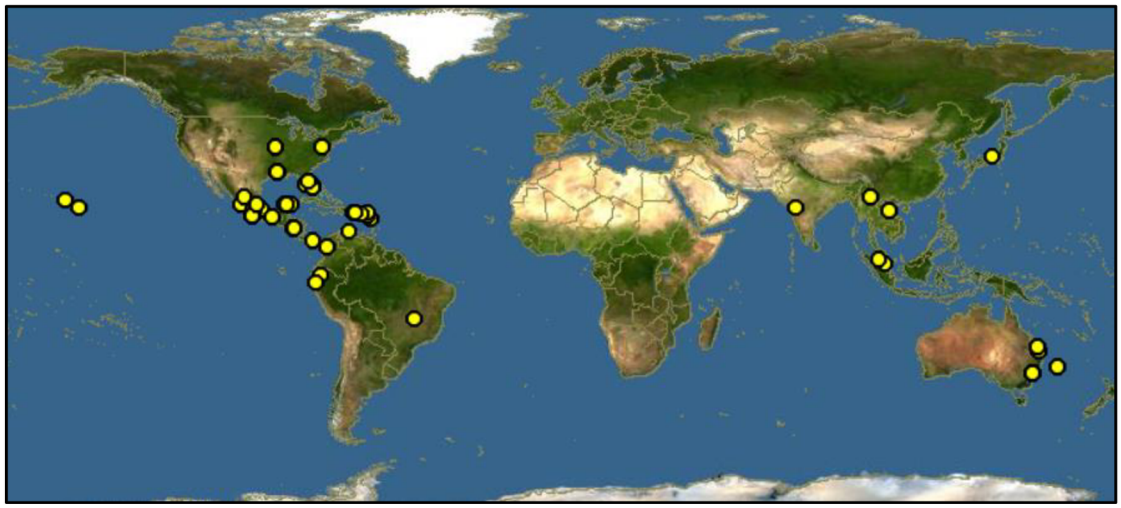

Figure 6. Distribution of *Callisia fragrans* in the world

Source: "Discover life" 2022

1.2.3.1. Botanical and morphological description

It is a diploid succulent herb growing up to 1,5 m (Figure 7). Stem is thick with markable nodes and producing long runners (stolons). The long stolons produced during the vegetation are rooting freely at the nodes (Celarier 1955; Hunt 1986; Nandikar et al. 2010). Leaves are alternate, parallel veined with markedly developed leaf sheath (Nandikar et al. 2010; López-ferrari et al. 2014).

Inflorescence (Figure 8) of *C. fragrans* is perched on the stem and pleasantly honey-like smells. Size of calyx leaves ranges between 3.5 - 5 × 1.5 - 2 mm and the crown leaves are white and have dimensions of 5 - 6 × 2.5 - 3.5 mm. The flower includes 6 stamens, which are long and more conspicuous than crown petals (Hunt 2001).

There are more resources presenting different chromosome numbers for *C. fragrans*. Somatic chromosome numbers were detected as 2n = 2x = 12 (Darlington 1929; Anderson & Sax 1936; Hunt 1986; Stuessy 1990; Saensouk & Saensouk 2020) and 2n = 2x = 18 (Nandikar et al. 2010). *C. fragrans* has genome with six pairs of medium sized chromosomes (Darlington 1929). Karyotype description as two chromosome pairs median or submedian and four with subterminal or terminal constrictions was reported by Anderson & Sax (1936). Saensouk & Saensouk (2020) reported karyotype as three pairs metacentric and three pairs subtelocentric chromosomes.

Figure 7. *C. fragrans* plant

Figure 8. *C. fragrans* inflorescence

1.2.3.2. Uses and chemical compounds

Callisia fragrans is often grown as an ornamental plant in gardens, in Thailand its leaves are used as a vegetable (Chumroenphat & Saensouk 2021). Most importantly it is used as a medicinal plant in folk medicine for treatment various diseases such as cardiovascular, oncological, gastrointestinal diseases, tuberculosis, arthritis, bronchial asthma, and infertility (Nandikar et al. 2010; Malakyan et al. 2015; Sohafy et al. 2020). It is known for its anti-inflammatory effects, which increase its use for treatment skin diseases, mucosal infections, healing bruins and wounds and fight against inflammation in folk medicine (Malakyan et al. 2015; Karapetyan 2020; Sohafy et al. 2020).

Pranskuniene et al. (2019) research found out that in folk medicine the leaves, which are 9 parts of the stem, load up in 40-degree alcohol are used for joint disorders, ethanol leaves extract is also used for improving immunity.

Different compounds in *C. fragrans* are reported to be present such as biologically active flavanoids and phytosteroids, phenolic compounds, amino acids, carbohydrates,

coumarins, vitamins, etc. (Chernenko et al. 2007; Karapetyan & Mairapetyan 2020). In a juice, which was made from fresh leaves and stolons, 7 essential aminoacids were found (Nikolaeva & Nikolaeva 2009). The high content of vitamin C, PP, and B2 and the mineral elements such as Ca, Mg, Fe, Cr, Ni, Si, P, Ba, Na, Mn, Cu, Zn and Al are found in the juice (Chernenko et al. 2007; Malakyan et al. 2015; Nikolaeva & Nikolaeva 2009).The content of vitamin C was observed by Karapetyan & Mairapetyan (2020), who found out that hydroponics grown plants contained 30 – 40 % higher content of ascorbic acid than plants which were grown in soil (Karapetyan & Mairapetyan 2020).

HPLC detected 12 compounds of which is importantly gallic acid, caffeic acid, chicoric acid, ferulic acid, quercetin and kaempferol. Of which gallic acid content was measured for 3.21 mg/g of dry juice (Olennikov et al. 2008).

Chumroenphat & Saensouk (2021) found out that *C. fragrans* has a high antioxidant activity which was determined by FRAP (104.7 mg FeSO4/g) DW and DPPH free radical scavenging assay (38.0 mg TE/g DW). Due to its antioxidant properties the radioprotective activity was tested. It was found out that water extract of *C. fragrans* could be effective radioprotector (Malakyan et al. 2015).

Valuable anti-inflammatory activity was detected in ethanol extract of aerial parts of *C. fragrans*. The dosage of 5 mg/kg p.o was 39.6 % more efficient than diclofenac sodium for treating inflammation (Sohafy et al. 2020).

Minh (2019) revealed that *C. fragrans* leaves are best to dry in microwave for production tea, which preserves the highest content of compounds (Minh 2019).

1.3. Plant breeding

Plant breeding is the process of developing the genetic and morphological appearance of plants, obtaining new varieties, or simply improving existing varieties. These varieties might be better adapted to any type of stress, such as drought, waterlogging or pH tolerance (Chloupek 2008). Plant breeding has positive results, but also a negative side effects. For example, newly obtained varieties may have for higher yields, larger organs, or biochemical compounds due to plant breeding methods. For example, the breeding for a high-yielding varieties can lead to susceptibility to pests and

diseases (Graman and Čurn, 1998). Many breeding methods can be used to obtain desired traits. There are conventional methods, for example hybridization (crossbreeding), mutagenesis and polyploidy breeding as well as nonconventional methods involving in vitro techniques, and gene manipulation (Allard 1960; Kuliffay & Svitič 1982; Graman & Čurn 1997).

The oldest method of plant breeding is selection, but it does not improve genetic variability. There are two categories of selection the natural and artificial selection. Natural selection is slow process that relies on the ability of plants to adapt to different conditions in order to survive. Long-term exposure to certain conditions can lead to plant adaptations, such as long-term drought, which in evolution can shorten reproduction times. This process is based on improving the biological qualities of plants. Breeders have been using artificial selection since the beginning of agriculture. This method is responsible for the emergence of the original varieties. The purpose of artificial selection is to either select for positive traits of interest in breeding, or to exclude negative traits that occurred during the breeding process (Allard 1960; Chloupek 2008; Graman & Čurn 1997).

The hybridization process improves the genetic variability of the breeding material. It is an effective and commonly used method in breeding process. This method began commonly used only after the rediscovery of Mendel's laws. Mendel the horticulturist and plant breeder, who tried to improve varieties through artificial pollination (Van Dijk et al. 2018). The purpose of the hybridization is to obtain hybrid offspring that are bearing the traits and characteristics of the parents used, combined in different ways. Parental plants are usually carefully selected in order to obtain conditional traits and combinations that can be described as newly bred characters. Parental components are usually intraspecific, but hybridization is possible even with interspecific and intergeneric parental components. Hybridization of unrelated species has its difficulties such as a sterility of F1 hybrids. Some species cannot even hybridize due to obstacles such as different flowering times, morphological and anatomical differences in plant reproductive organs, and different chromosome numbers and other barriers (Allard 1960; Graman & Čurn 1997; Shmeit 2015)

The most common type of hybridization is heterogeneous breeding which is used for breeding of F1 hybrids. These hybrids are important due to their properties such as increased vitality, disease resistance or increased yields. The most efficient in breeding F1 hybrids are homozygote parental components. The usage of this parental combination produces uniform F1 generation with the best properties (Kuliffay & Svitič 1982; Graman & Čurn 1997; Chloupek 2008)

Mutations in plants ensue spontaneously but can be also induced artificially, which is mostly used by breeders. Mutagenesis has it begins from the 1950´s when the ionizing radiation was used to induce these changes. Inducing of mutations expands the genetic variability of the breeding material. Single gene mutations and genomic mutations are the most important changes used in plant breeding. The mutagens used for induction DNA changes are of physical origin (for example: UV, roentgen, gamma radiation etc.) or chemo-mutagens (for example: alkaloids, peroxides, purines, EMS etc.). Mutagenesis is mostly used in self-fertile plants and plants which are propagated vegetatively (Allard 1960; Kuliffay & Svitič 1982; Graman & Čurn 1997).

First successful polyploidy was performed by Murashige and Nakano, who induced polyploidy in tobacco (Murashige and Nakano 1966). It is genomic mutation in plant cells, that raised the sets of chromosomes, caused by abiotic stress or other factors that inhibits mitotic spindle function (Shmeit 2015). Polyploidy causes many changes such as increase of the vegetative parts, increase the yield but could reduce the seeds production (Graman & Čurn 1997; Chloupek 2008; Salma et al. 2017).

Non-conventional methods are used in last few decades. It includes for example *in vitro* techniques and gene manipulations (Graman & Čurn 1997).

In vitro propagation is method which is used to multiplicate clones. The obtained material is 100% identical to the mother plant. The most used and commercially successful is meristematic micropropagation. It is used for propagation of for example sterile hybrids of bananas, disease free potato seedling tubers, or multiplication valuable genotypes and ornamental plants etc (Graman & Čurn 1997; Scaramuzzi et al. 2000).

Gene engineering is nowadays widely studied. Using its methods is possible to insert concrete gene into the plant DNA to obtain new genotype, where is added that concrete trait. These genes may encode properties such as for example resistance against

herbicides, pathogens or natural conditions such as drought or salinity or even improve nutritional value (Kuliffay & Svitič 1982; Graman & Čurn 1997). Trait of β carotene was added into the rice genome to prevents the vitamin A malnutrition in people which diet consist mostly of rice (Mallikarjuna Swamy et al. 2021). Transgenic plants such as corn, wheat, rice or soja are recently developed and used (Kuliffay & Svitič 1982; Graman & Čurn 1997; Mallikarjuna Swamy et al. 2021).

1.4. Polyploidy

Polyploidy occurs naturally, via plant adaptation to environmental changes. This is a slow but frequent process. Polyploidy is defined as three or more complete sets of chromosomes in genome which stimulated variance within the plant kingdom evolution (Shaikh et al. 2021). It is said that about 30 – 35 % of angiosperms and 70 % of grasses are polyploid (Graman & Čurn 1997).

Polyploidy discovered in 1907 may also be the result of artificial induction using antimitotic agents (Salma et al. 2017; Eng & Ho 2019). Artificial polyploidization is more effective and rapidly induced with antimitotic agents such as colchicine, oryzalin, trifluralin, pronamide and amiprophosmethyl. Induced polyploidy is more effective than mutagenesis because it effectively affects the entire genome. All genomic mutations produce more phenotypes than mutagenesis (Eng & Ho 2019).

Polyploidy is used for breeding plants which are cross pollinated, with lower chromosomal number, vegetatively propagated or produce high number of seeds. There are two results of induce polyploidy, autopolyploidy and allopolyploidy. Autopolyploidy is the result of genome multiplication whereas allopolyploids arise through hybridization of species with different ploidy levels (Kuliffay & Svitič 1982; Graman & Čurn 1997).

Polyploid plants usually have larger vegetative organs, inflorescences, and fruits. Usually, the stomata and chloroplast size increase along with the pollen cells. A similar feature that unites all polyploid plants is the reduced seed content (Graman & Čurn 1997). Polyploids can thrive in extreme environments, including dry climates, subarctic and high-altitude regions. It is presumed that polyploid species can thrive much more effectively than the diploids due to their vigorous morphological, physiological and

growth differences that may be the reason for their higher stress resistance (Salma et al. 2017). The polyploidy breeding of medicinal plant is efficient in obtaining species with higher biomass which contains higher metabolites content and active ingredients (Salma et al. 2017). For example, higher essential oil content was obtained in *Chamaemelum nobile* (Tsuro et al. 2016). *Atropa belladonna* autotetraploid contained 1.5 times higher number of alkaloids than the diploid plant (Salma et al. 2017). Higher phenolic acids content was observed in *Echinacea purpurea* (Mei et al. 2020), *Salvia officinalis* (Gao et al. 1996; Kaensaksiri et al. 2011; Hassanzadeh et al. 2020).

The effective induction of polyploidy depends on a different factor, such as explant types, antimitotic agents and its concentrations and duration times (Salma et al. 2017). There are a lot of antimitotic agents belonging into the diverse chemical groups such as dinitroanilines (trifluralin and oryzalin), benzamides (pronamide), phosphorothioamidates (amiprophos-methyl), carbamates (chlorpropham, isopropyl N- 3-chlorophenyl carbamate) and others (Salma et al. 2017). The most frequently used antimitotic agents are colchicine, oryzalin and trifluralin (Eng & Ho 2019).

Colchicine is a naturally occurring alkaloid which is extracted from *Colchicum autumnale* plants. This plant belongs into the family Colchicaceae. Its mitotic effect was discovered by Allen in 1936 on animal tissue. Experiment with plant treatment with colchicine first attempted Eigsti in 1938 (Eng & Ho 2019). Till 1960 the polyploidy treatment was experimented only in *ex vitro* conditions. This agent is used for the longest time, from the beginning of polyploidy experiments. Colchicine prevents chromosome dislocation to the poles of the cells (Eng & Ho 2019; Kaensaksiri et al. 2011; Horký 2013). It also exhibits side effects such as abnormal growth, sterility, and chromosome aberrations. Colchicine is highly toxic to humans, causing death at doses 0.8 mg/kg (Eng & Ho 2019). This antimitotic agent is nowadays being replaced by herbicides (oryzalin, trifluralin etc.) due to their better affinity to tubulin and lower toxicity to human (Eng & Ho 2019; Zeng et al. 2019).

Nowadays the mostly used antimitotic agents are dinitroaniline herbicides. These substances belong to the group of emergent herbicides that cause gross morphological abnormalities in plants, especially in areas with high meristematic activity. These substances inhibit mitosis in the plant cells, due to its excellent ability to bind to tubulin.

Due to this ability, lower doses are needed to induce polyploidy than when colchicine was used. Oryzalin and trifluralin are potent antimitotic agents, which become a standard preference against colchicine (Hess & Bayer 1977; Morejohn et al. 1987; Viehmannová et al. 2009; Eng & Ho 2019). Greplova et al. (2009) and Viehmannová et al. (2009) reported oryzalin more effective in comparison with colchicine in inducing polyploids in *Solanum* species. Oryzalin was also more efficient in inducing of polyploidy in *Spathiphyllum wallisii (*Eeckhaut et al. 2004) and *Agastache foeniculum* (Talebi et al. 2017).

1.5. Molecular markers

Molecular markers are nowadays an important tool for detecting polymorphism in genetic studies, to track locus and genome regions. It can be applied to many aspects of breeding strategies. They allow the breeders to characterize genetic diversity due to its great detection of high levels of polymorphism. A significant advantage of molecular markers over biochemical and morphological markers is independence from external conditions, detectability in all tissues and information on polymorphism in coding and non-coding regions of DNA (Singh et al. 2008; Kumar et al. 2009; Vanžurová 2011).

There is a possibility to detect the polymorphism in or between the group of species or populations. These markers are nucleotide sequences corresponding to a specific location in the genome. They are used to mark the possible location of the genes coding the concrete traits on chromosome. These genes could be subsequently isolated and characterised (Singh et al. 2008; Kumar et al. 2009; Erdinc et al. 2021).

DNA polymorphism could be detected by various techniques. One of the most used is the gel electrophoresis. There are two gel matrices which can be used for separation of the molecules, agarose, and polyacrylamide. The ability of separation of molecules in the gel depends to its concentration (Singh et al. 2008). The negative electric charge that DNA has is the basis of this method. The DNA is poured into a gel near the negative pole, and it penetrates to the positive pole. Due to a type of procedure and range of fragments the type of gel needs to be appropriately chosen. In procedures such as RFLP and RAPD agarose is usually used. Due to microsatellites and AFLP method produce

smaller fragments, it is better to use polyacrylamide (Kumar et al. 2009; Singh et al. 2008).

DNA based markers could be divided into two types, non-PCR based (RFLP) and PCR based markers (RAPD, RAMP, iPBS, AFLP, SSR, SNP etc.) First DNA markers were used to study genetic diversity of crops (Kumar et al. 2009; Vanžurová 2011).

CDDP markers are a PCR technique with non-specific primer sequences. These markers are used to amplify regions by single primer, where the primer is used as a forward and reverse prime. Gene-specific primers targets conserved regions of DNA within genes and in plant genetics for searching for QLT (Quantitative trait locus) across plant species. Marker and gene (or QTL) recombination level is in comparison with "indirect random markers" such as RAPDs, ISSRs, or SSRs lower. (Collard & Mackill 2009).

The iPBS method is detecting specific target sequences in the genome. The iPBS technique proved to be an efficient fingerprinting technology when there is no prior sequence knowledge. It is set up on the presence of complementary tRNA, in LTR retrotransposons, as a reverse transcriptase primer binding site. There is only limited amount of tRNA which tie up to PBS primers. Subsequently a specific family of LTR retrotransposons can be determined by sequencing (Kalendar et al. 2010; Vanžurová 2011; Erdinc et al. 2021).

2. Aims of the Thesis

The main objective of this thesis was to evaluate the morphological and molecular variation between autotetraploid (2n=24) and diploid (2n=12) plants of *Callisia fragrans* (Lindl.) Woodson. Autotetraploids (new synthetic genotypes) were obtained by technology of induced mitotic polyploidy *in vitro* using oryzalin as an antimitotic agent.

The aim of this work was established according to the following hypotheses:

H1: *In vitro* somatic polyploidy is effective method to obtained new synthetic genotypes.

H2: By somatic polyploidization can be achieved new genotypes with different morphological, biochemical and molecular properties in *C. fragrans.*

3. Methods

This research relates to my bachelor thesis, named In vitro induction of polyploidy in *Callisia fragrans* (Lindl.) Woodson. This thesis follows up the research and continue with evaluation of obtained plants and further qualities.

3.1. Plant material and culture establishment

Callisia fragrans diploid plants (2n=2x=12) were grown in greenhouse condition (average temperature 22.5 °C; relative air humidity 70 % - 80 %) in plastic containers (9 × 9 cm). After 4 weeks of cultivation the stolons started to appear. At that development stage, plants were transplanted into larger containers (15 cm in diameter). Over the next eight weeks of cultivation the stolons grew up to the 60 cm in length. Thereafter, nodes of stolons were introduced into *in vitro* conditions by sterile nodal buds and then were propagated on MS media (Murashige & Skoog 1962) supplemented with 1 mg/l BAP and 0.1 mg/l NAA in Erlenmeyer flasks. Explants were cultivated under controlled conditions (day/night temperature 25 °C/20 °C and day/night photoperiod 16h/8h with the light intensity of 3000 lx.

3.2. Polyploidy induction

The chromosome doubling was induced in a total 180 plants and were divided into Erlenmeyer flasks in the quantity of 5 plants in one flask. Oryzalin (antimitotic agent) was added to MS growth media in concentrations 1, 5, 10 μM/l. The treatment time was setted for 4 and 8 weeks. For every variant (concentration x treatment time) 30 plants were used but during the treatment some plants started to created offshoots. After the treatments, all obtained plants were washed in distilled water and transported on oryzalin-free MS media.

3.3. Ploidy level determination

The ploidy level was determined via Flow cytometry and by chromosome counting in metaphase cells.

3.3.1. Flow cytometry

Samples were prepared and ploidy level determined according to basic protocol (Zahumenická et al. 2018). Samples were taken from all treated, and control plants after 2 months of cultivation on oryzalin-free MS media. The sample (approx. 1 ×1 cm wide) was placed in Petri dish, covered by 1 ml Otto I (Tween 20 + citric acid) and cut into small fragments, for disruption of the tissue. The suspension was filtered through a nylon filter (50 µm) into a tiny tube. The 1 ml Otto II + DAPI solution (Na_2HPO_4 . 12 H_2O + 4',6-diamidin-2-fenylindol, fluorescence colouring) was added to the suspension.

3.3.2. Chromosome counting

Fresh roots tips (1 cm approx.) of Diploid and Tetraploid plants were taken between 7-8 o'clock in the morning and kept submerged in a saturated solution of Paradichlorobenzene for 3 hours. 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 hour 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 minutes followed by washing three times and staining using Schiff ś reagent for 1 hour. Roots tips were removed from the Schiff ś 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 100X magnification (Tomaskova & Dobiasova 1995).

3.4. Plant transfer into ex-vitro conditions

The newly obtained genotypes and the control plant maintained in *in vitro* conditions for a month, after that were removed from Erlenmeyers flasks. Plants of the same age with well-developed root system were transferred into the pots. The roots were washed from remnants of medium in lukewarm water and potted into a gardening substrate Agro profi RS 1 (composition: 70 % white peat; 30 % black peat; 20 kg / m^3 bentonite; 1.1kg / m^3 N, P, K (14 %, 16 %, 18 %) + ME; 150 g / m^3 Micromax Premium; 100 ml / m^3 wetting agent) with the addition of fertilizer Osmocote Exact standard (3 kg / m^3, gradual release 8-9 months) into pots 9×9 cm. In order to create suitable conditions for the transfer of plants were newly potted plants covered with plastic foil for 1 week to ensure sufficient moisture. The plants were then grown in a greenhouse condition (an average temperature 22.5 °C). Once in 2 weeks the morphological differences were evaluated and measured. The morphological differences were monitored and measured for more than a year till the plants bloom out.

3.5. DNA extraction

Total genomic DNA of *C. fragrans* was extracted from plants grown in pots. A mixture of leaf tissue was prepared from 10 randomly chosen discs with 1 cm in diameter, obtained from at least 5 leaves. The surface of the discs was disinfected slightly and processed. DNA was extracted by GeneJET plant DNA purification kit (ThermoScientific) according to the instructions of the manufacturer. Quantity and quality of extracted DNA were measured by Nanodrop P500 (Implen) and its functionality in PCRs was proved by ITS analysis using the universal barcoding primers (White et al. 1990)

3.6. iPBS analysis

Different primers (Kalendar et al. 2010) for inter primer binding site polymorphism analysis (iPBS) were firstly screened to be applicable for *C. fragrans* fingerprints generating and subsequently six primers were chosen for the final analysis. PCRs were prepared by EliZyme Robust HS (Elizabeth Pharmacon) together with the 800

nM of iPBS primer and the 50 ng of DNA in each reaction. PCR conditions of the iPBS were as follows: 95 °C for 5 minutes; 45 cycles of 95 °C for 1 minute, 55 °C for 1 minute, 72 °C for 2 minutes; and 72 °C for 10 minutes. The PCRs were repeated twice.

3.7. CDDP analysis

Conserved DNA-Derived Polymorphism (CDDP) method was used to analyse the variability of *C. fragrans* with the primers reported by Collard and Mackill (2009). PCRs were prepared by EliZyme Robust HS (Elizabeth Pharmacon) together with the 400 nM of each primer and the 50 ng of DNA in each reaction. Time and temperature profiling of the PCRs was as follow: 95 °C for 5 minutes; 40 cycles of 95 °C for 45 seconds, 54 °C for 45 second, 72 °C for 90 seconds; and 72 °C for 10 minutes. The PCRs were repeated twice.

3.8. DNA fingerprints data analysis

The obtained iPBS and CDDP fingerprints data were analysed using the 2 % agarose electrophoresis and the reproducible generated profiles were converted into 0-1 binary matrices. The UPGMA analysis was performed, and the Dice coefficient of genetic similarity (1945) was used for dendrograms construction. The dendrograms were prepared in the DendroUPGMA free software (http://genomes.urv.cat/UPGMA/).

3.9. Mineralization and ICP-OES elemental analysis of micro and macronutrients

Approximately 0.25 g of each sample was weighed on analytical balances ABT-120/5DW (Kern & Sohn) and transferred to PTFE mineralization tubes. Mineralization was performed in pressure microwave digestion on EthosOne (Milestone) in 5 mL concentrated nitric acid 69 % and 1 mL of 30 % hydrogen peroxide with 2 mL of ddH_2O. The obtained digestate was filtered through Filtrak 390 (Munktell & Filtrak). Elemental analysis was performed in Agilent 720 ICP-OES spectrometer (Agilent technologies Inc.) with axial plasma configuration and with autosampler SPS-3 (Agilent Technologies).

3.10. Statistical evaluation

Statistical analysis of data from the morphological evaluation of tetraploid and diploid (control) plants, grown in greenhouse conditions, were performed using statistical software Statistica®. The Kruskal-Wallis test was used to compare the differences between treatments. For the test, differences were considered as significant at $P < 0.05$.

4. Results

4.1. Induction of polyploidy and detection of ploidy level

A total 180 plants were exposed to oryzalin using three concentrations during two treatment times. 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. Results are summarized in Table 2. All treated plants were tested by Flow cytometry (Figure 9; Figure 10). 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 chromosome in the somatic cells (Figure 10; Figure 11). In total, six autotetraploid and two mixoploid plants were obtained from diploid mother plants All autotetraploid genotypes were obtained from 5 µM/8-week treatment. Mixoploid plants were regenerated from 5 µM/4-week and 10 µM/8-week treatment. Autotetraploid plants were marked as P1 - P6 and mixoploid plants as M1 - M2. Genotype P6 was not viable, exhibited slower growth and inability to create offshoots. Obtained results confirms the first hypothesis that *in vitro* somatic polyploidy is effective method to obtained new synthetic genotypes. All induced autotetraploid plants were transferred into *ex vitro* conditions, the survival rate was 100 %.

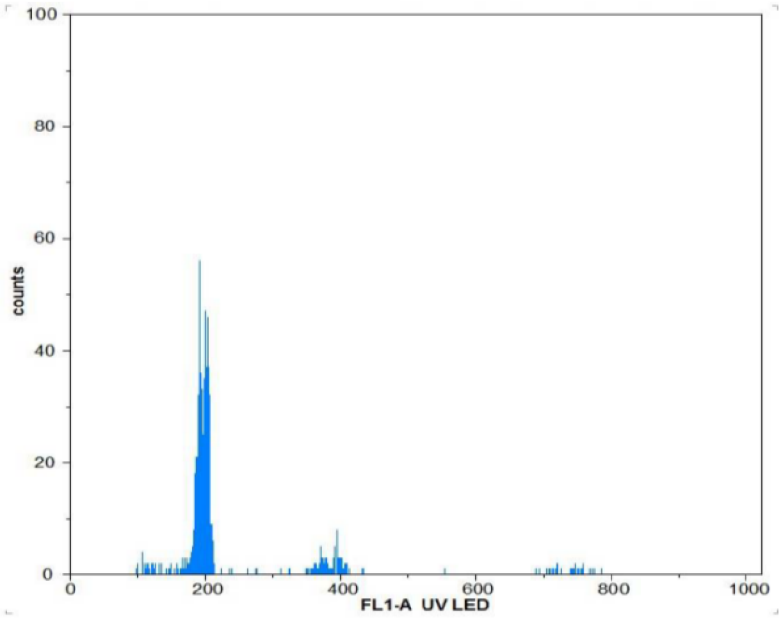

Figure 9. Flow cytometry analysis of control plant. *C. fragrans*: histogram of control diploid (2x) plant

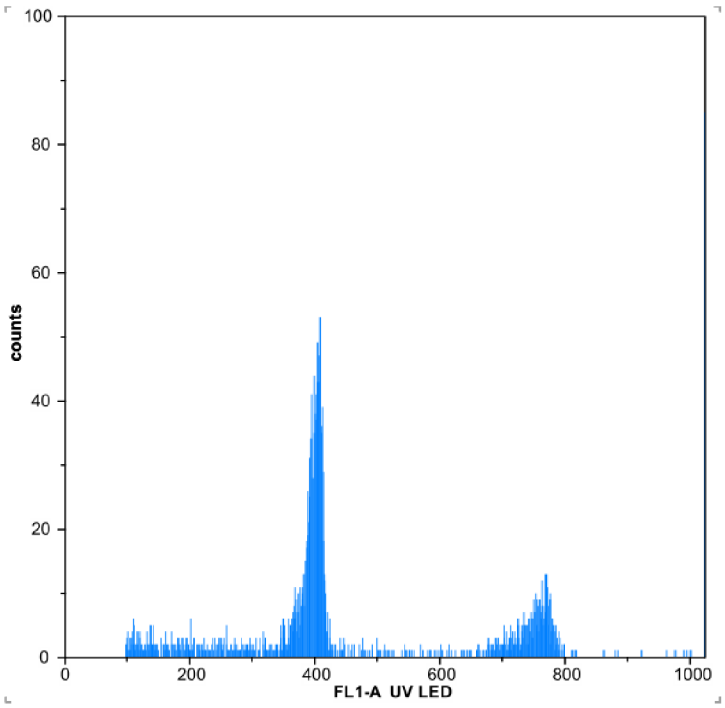

Figure 10. Flow cytometry analysis of oryzalin treated plant. *C. fragrans*: histogram of tetraploid (4x) plant

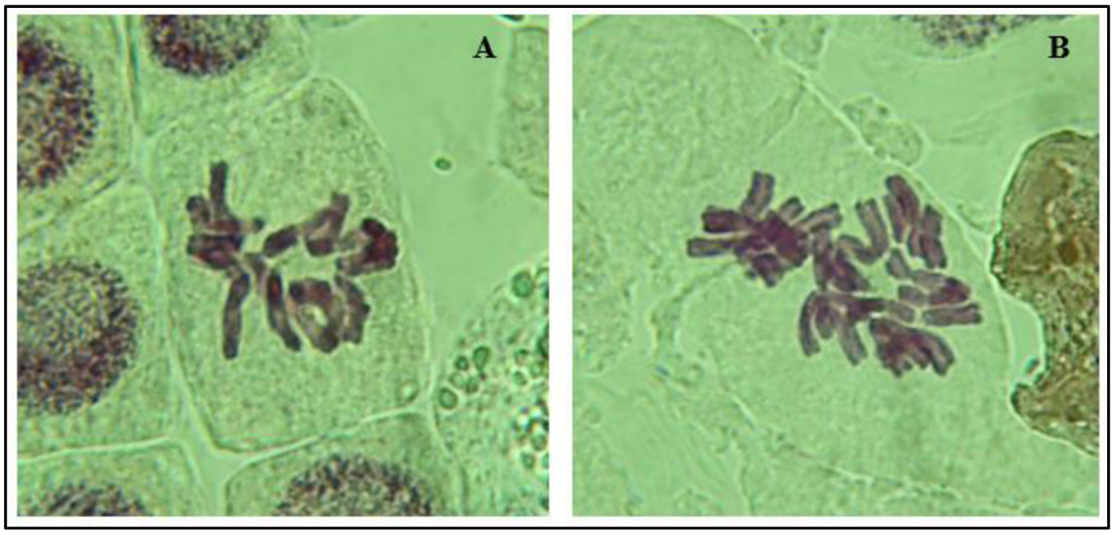

Figure 11. Chromosome counting of *C. fragrans*. **A.** Chromosome numbers in diploid *C. fragrans* (2n=2x=12). **B**. Chromosome number in induced autotetraploid *C. fragrans* (2n=4x=24)

Table 2: Induced polyploidy of *C. fragrans* by oryzalin treatment

Concentration (µM/l)	**Duration (weeks)**	**Number of plants**	**Number of plants after treatment**	**Survival rate (%)**	**Mixoploid plants**	**Autotetraploid 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**	

4.2. Morphological differences between diploid and the tetraploid genotypes

Polyploid plants exhibited notable morphological differences in comparison with control genotype. The evaluation of growth variability is summarized in the Table 2 and 3. Significantly reduced number of leaves were detected in P3, P4, M1 and M2 genotypes. Statistically there was a significant difference in P3 and M1 genotype which generated significantly (about 2 cm) longer leaves (in average 31.34 ± 0.82 and 32.73 ± 1.61 cm, respectively) than control (30.87 ± 0.42 cm). This difference is noted in Figure 12. In the contrary, genotype P4 exhibited significantly the shortest leaves (22.35 ± 0.94 cm).

Autotetraploid plants were characterized by more compact growth compared to control plants. The most compact growth was observed in mixoploid M1 and M2 genotype (46 ± 7.76 and 46 ± 7.75 cm). Genotype P4 had significantly lower number of internodes (11.9 ± 1.37) than the control plant (16.3 ± 1.7) (Table 3; Appendix 3, 4).

All affected plants produced shorter stolons. The longest stolons were observed in the control variety (Figure 13; Appendix 2, 4). The highest number of stolons (6 stolons) were counted in M2 genotype (Figure 13). There were differences in the number of stolons between genotypes and the control plant, but it was not significant.

After 2 months of cultivation, the fresh biomass (aerial and underground parts) was weighed up. There was a significant increase in fresh biomass in genotype P3. The weight of plants raised up by 50.6 % on average. There were differences in the weight of biomass between other genotypes and the control plant, but it was not significant (Table 3.).

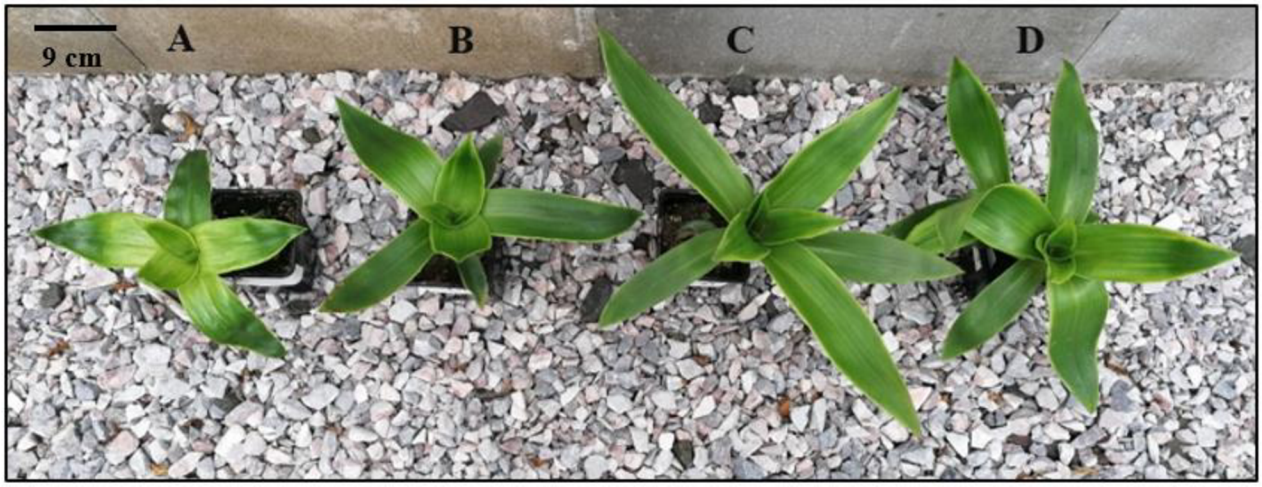

Figure 12. *C. fragrans* plants in *ex-vitro* conditions, 4 weeks after transfer: **A.** Control plant; **B.** Autotetraploid genotype P2; **C.** Autotetraploid genotype P3; **D.** Mixoploid genotype M1

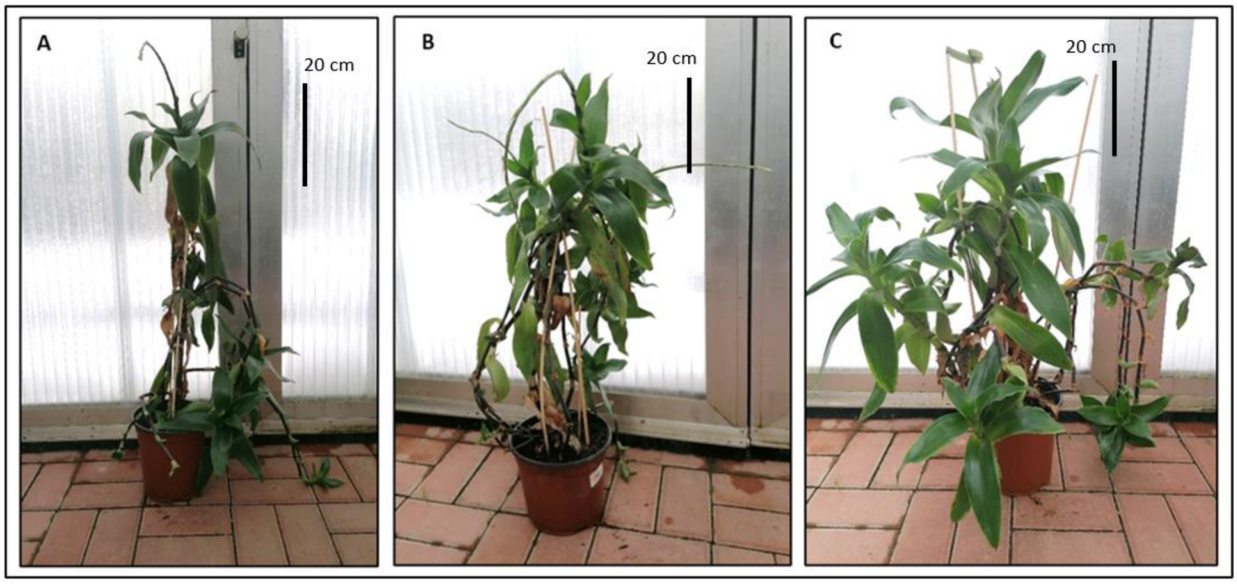

Figure 13. *C. fragrans* plants in *ex-vitro* conditions, 11 months old plants, starting to produce flower buds: **A.** Control plant; **B.** Autotetraploid genotype P4; **C.** Mixoploid genotype M2

Table 3. Morphological characteristics of diploid and tetraploid plants cultivated in pots

Variety	Average number of internodes	Average number of stolons	Average number of leaves	Height of plant (cm)	Average longines of stolons (cm)	Average number of internodes on stolon	Leave length (cm)	Fresh biomass (g)
Control	16.3 ± 1.7 [a]	5.5 ± 1.35 [a]	23.7 ± 2.26 [a]	64 ± 6.58 [a]	76.1 ± 10.04 [b]	10.33 ± 0.92 [a]	30.87 ± 0.42 [a]	67 ± 14.09 [a]
P1	14.8 ± 2.15 [a]	4.4 ± 1.17 [a]	22 ± 1.76 [a]	67.5 ± 10.61 [a]	54.95 ± 10.8 [a]	9.51 ± 0.86 [a]	28.69 ± 0.88 [a]	76.5 ± 10.83 [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 [a b]	6.92 ± 2.3 [b]	31.86 ± 1.57 [a]	83.2 ± 14.02 [a]
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 [a b]	9.33 ± 0.54 [a]	31.34 ± 0.82 [b]	132.4 ± 10.37 [b]
P4	11.9 ± 1.37 [b]	4.5 ± 0.53 [a]	17 ± 0.94 [b]	62 ± 10.33 [a]	61.48 ± 9.92 [a b]	8.19 ± 0.94 [c]	22.35 ± 0.94 [d]	71 ± 30.46 [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]	32.7 ± 1.14 [a]	82.3 ± 7.86 [a]
M1	12.3 ± 1.9 [b]	5.2 ± 0.42 [a]	18.8 ± 3.36 [b]	46 ± 7.76 [b]	56.86 ± 15.48 [a]	8.62 ± 1.35 [a]	32.73 ± 1.61 [c]	77.3 ± 15.09 [a]
M2	12.2 ± 1.81 [b]	6 ± 1.25 [a]	18.2 ± 2.49 [b]	46 ± 7.75 [b]	57.38 ± 13.73 [a]	8.82 ± 1.21 [a]	24.47 ± 1.48 [d]	76.1 ± 11.02 [a]

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

The polyploid plants were characterized by a larger flower size. For example, autotetraploid P3 when compared with diploid showed significant accrue in size in diameter (49.45 %). Genotype P3 exhibited longer and wider flower petals (Table 3; Figure 14). The longest flower petals were measured in P1 genotype (6.045 ± 1.01 mm; Appendix 5). Genotypes P2, M1 and M2 did not bloom at all. In other genotypes a significant variation was observed. Control genotype, P1 and P3 started flowering two week earlier than P4 and P5. After 11 months of cultivation cultivated plants started to produce flower clusters.

The results confirm the second hypothesis that newly obtained autotetraploid genotypes are morphologically, biochemically, and molecularly various from the diploid plant.

Figure 14. Morphological variation between diploid (Control) and autotetraploid (P3) flowers of *C. fragrans* cultivated in greenhouse conditions

Table 4. Morphological characteristics of flowers of diploid and autotetraploid plants cultivated in pots.

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

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

4.3. Nutrient content

The nutrient content analysis detected variance in the content of macro and micronutrients. Potassium showed an almost 100% increase in all genotypes. The Calcium content increased only in P1 genotype. The other genotypes have Calcium content reduced in comparison with diploid plants. Sodium content in autotetraploid plants was evaluated as six times higher (4-9×). Summary of nutrient content is marked in Table 5.

Table 5. Macronutrient, micronutrient and fragment compounds analysed from leaves of diploid, autotetraploid and mixoploid *C. fragrans* plants (mg.kg^{-1}).

Nutrient	C. fragrans 2x	C. fragrans 4x P1	C. fragrans 4x P2	C. fragrans 4x P3	C. fragrans 4x P4	C. fragrans 4x P5	C. fragrans mixoploid 4x/2x M1	C. fragrans mixoploid 4x/2x M2
K	15946.00	30947.60	37300.90	30027.00	> 39032.40	39032.40	33307.4	26330.20
Ca	23147.90	25688.50	8858.10	13760.30	13112.50	17225.90	17323.2	14787.40
Mg	12745.60	14280.50	4878.00	8026.50	6877.20	10310.70	9350.7	8513.40
Na	194.40	1365.20	1745.40	1297.50	1702.70	1399.80	1349.3	920.70
Fe	81.92	74.28	90.80	57.97	73.60	82.08	92.63	93.74
Mn	101.70	107.40	142.60	89.70	118.60	78.80	186.2	135.00
Zn	48.51	43.45	53.88	37.07	55.93	44.16	50.66	39.45
Cu	26.97	34.38	54.16	25.80	32.34	24.99	43.18	41.83
Cr	0.151	0.17	0.16	0.11	0.18	0.08	0.16	0.20
Mo	0.39	0.51	0.45	1.01	0.14	ND	0.68	1.37
Co	ND	0.29	ND	0.20	ND	0.11	0.34	ND
Ag	ND	0.07	ND	ND	ND	0.07	0.11	0.02
Sr	48.97	43.25	53.88	38.15	55.93	45.94	50.66	38.16
Sb	ND	1.92	1.9	1.71	1.43	0.9	0.46	ND
Li	12.19	8.02	2.41	7.64	4.05	4.83	6.12	9.68
Ba	8.42	7.15	3.07	5.28	4.96	5.23	5.3	4.72
Al	35.92	11.43	17.34	16.26	13.98	13.83	18.98	13.52
Other	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

4.4. iPBS analysis (Inter Primer Binding Site Polymorphism)

A total of 21 different iPBS primers were screened to obtain polymorphic fragments (Figure 15; Kalendar et al. 2010). Six of them generated no amplicons and six others were used for the analysis as they provided sufficient number of iPBS amplicons (primers 1838, 1846, 1882, 2270, 1897 and Frodo2).

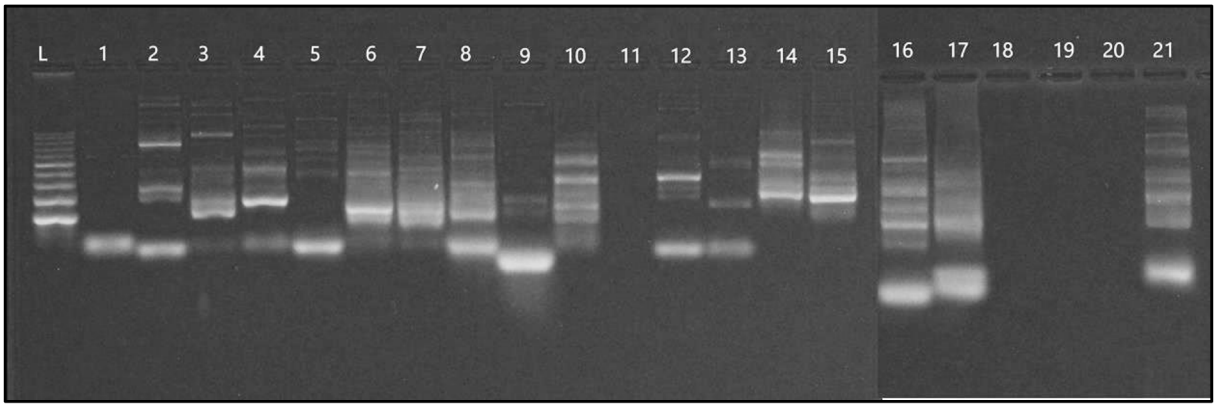

Figure 15. Results of the iPBS primers screening.

Primer 1882 generated monomorphic profile for all of the analysed polyploid *C. fragrans* accessions (P1 - P4, M1 - M2) with two (80 bp plus 90 bp) amplicon deletions in the control plant. In the control plant, 9 fragments were amplified and in the polyploid plants accessions, 11 iPBS fragments were amplified.

A completely monomorphic iPBS profile was obtained for the primer 1897 with the number of generated amplicons – 7. Primer Frodo2 generated different amplified iPBS profile for the accession M2, where deletions of short fragments were obtained, but all of the other samples have 12 iPBS fragments per accesion.

Three of the used iPBS primers provided polymorphic profiles among analysed *C. fragrans* plants. Primer 1838 generated 88 % polymorphism and varied from 3 (control) up to the 8 (P4) amplicons per accession. Insertion of the 260 bp fragments were obtained 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 the 990 bp.

Primer 2270 was characterised by 55 % of polymorphism but provided a different iPBS profile for the control *C. fragrans* plant, where two deletions were obtained. Profiles of M1-P5 were monomorphic. The number of generated amplicons were 5 for control plants and 7 for the polyploid plants.

Primer 1846 provided 87.5 % of polymorphism and was the most variable among the used iPBS primers. No specific pattern was found between the analysed genotypes and control plant of *C. fragrans*. They were not especially different inf their iPBS profile, but here, the most amplicons were obtained (11) The rest of the samples provided in iPBS from 4 up to the 9 amplicons. Genotypes M1, P2 and P4 have similar, but not the same profiles and plants P1 and P5 generated the same iPBS profiles (Figure 16).

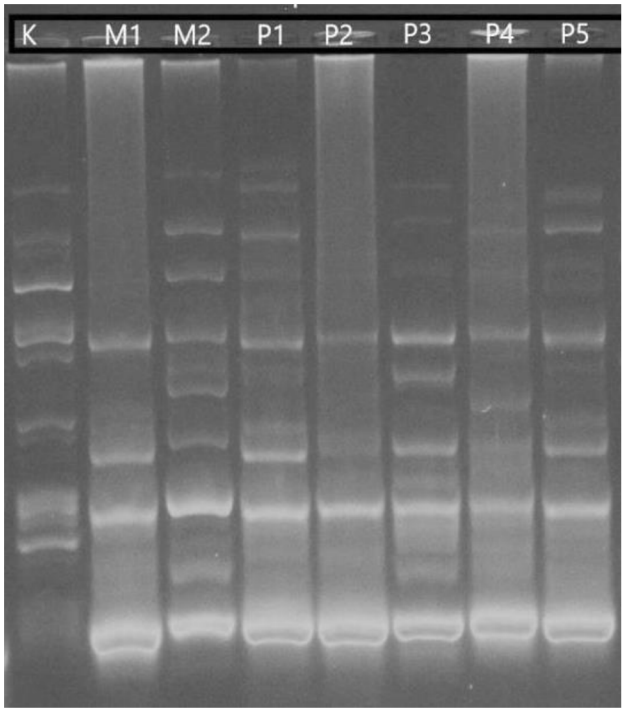

Fig. 16 iPBS profile of *C. fragrans* accession generated by primer 1845.

Dendrogram (Figure 17) was constructed for the analysed *C. fragrans* plants based on the results of iPBS fingerprinting. Three main branches were generated with iPBS profile from the most distinct control plant in comparison with the rest of the control

plants. The P1 - P5 plant were divided into two smaller subclusters with the same profile for P1 and P2.

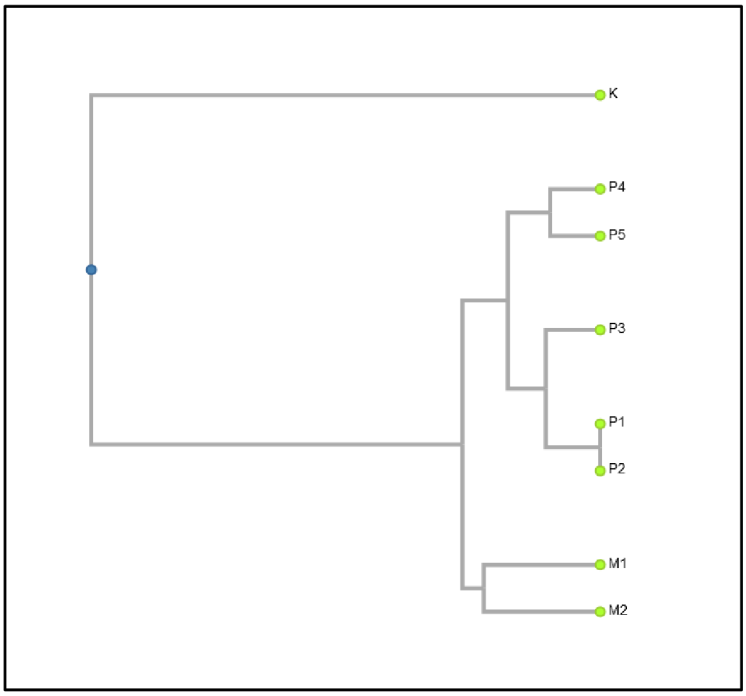

Figure 17: Dendrogram of analysed *C. fragrans* plants.

4.5. CDDP analysis (Conserved DNA-derived polymorphism)

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

Primer combination F+R1 provided a 430 bp insertion in the profile of 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 490 bp locus was generated and locus 510 bp has stronger amplification when compared with the other analysed *C. fragrans* accessions.

Primer combination F+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 in approximately 130 bp.

Primer combination FplusR2b provided differentiated fingerprints for control plant and P1. The control plant was typical by insertion of 240 bp locus and deletion of 260 bp locus. M plants and P2-P5 plants have monomorphic profiles and P1 plant is different from them by shift of the CDDP fingerprints and insertion of loci 130 bp and 140 bp.

Primer combination F+R3a generated monomorphic profiles for both, M and P plant, too. The control plant is different from them by insertion of loci 150 bp and 370 bp.

Primer combination F+R3b provided a completely monomorphic profile off all analysed *C. fragrans* plants (new genotypes and control plant).

Dendrogram (Figure 18) was constructed for the analysed *C. fragrans* plants based on the results of CDDP fingerprinting. Three main branches were generated with CDDP profile from the most distinct control plant in comparison with the rest of the analysed 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.

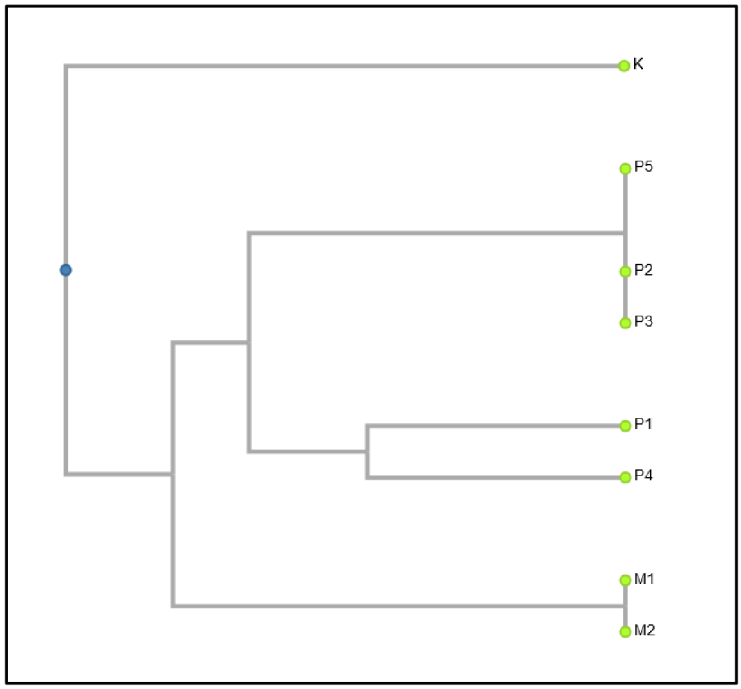

Fig. 18 Dendrogram of analysed *C. fragrans* plants based on CDDP results.

5. Discussion

Currently, there is no information about *in vitro* induced somatic polyploidy in *C. fragrans* nor in other species from *Callisia* genus. The only research done, used colchicine on roots in *in vivo* conditions in last century. (Roy 1973; Singh & Roy 1990). Experiment of *in vitro* induced somatic polyploidy in *C. fragrans* is done for the first time. Recent articles reports that *in vitro* induced somatic polyploidy is often used for breeding medicinal plants from other genuses and families such as *Allium cepa* (Yun et al. 2021), *Moringa oleifera* (Ridwan & Witjaksono 2020, *Chamaemelum nobile* (Tsuro et al. 2016).) and *Echinacea purpurea* (Mei et al. 2020). As an antimitotic agents oryzalin and colchicine are effectively used for obtaining new genotypes. Nevertheless, in medicinal plants it is reported that oryzalin have become a standard preference against colchicine due to its lower toxicity (Salma et al. 2017; Niazian & Nalousi 2020). Oryzalin seems like an effective antimitotic agent for in *vitro* induced polyploidy of *C. fragrans*. It is also used in breeding many medicinal plants for example *Calendula officinalis* (Esmaeili et al. 2020) and *Cnidium officinale* (Kim et al. 2021). Positive result using oryzalin were obtained Shmeit et al. (2020) *in Thymus vulgaris*. Oryzalin also worked effectively for the chromosome doubling of *Scutellaria barbata* (Yue et al. 2011) and *Rubus sanctus* (Sabooni et al. 2022). Eeckhaut et al. (2004) described that using oryzalin for polyploidization raised efficiency of obtaining polyploids by 5 %. Oryzalin is more effective than colchicine in genome doubling of *Smallanthus sonchifolius* (Viehmannová et al. 2009), *Solanum* species (Greplová et al. 2009) and *Agastache foeniculum* (Talebi et al. 2017).

Oryzalin added into media seems to be effective antimitotic agent for polyploidization of *C. fragrans*. The addition of 5 µM/l of oryzalin directly into the media for 8 weeks treatment time seems the most efficient 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. The addition of oryzalin in media was successful for polyploidization of *Anemone sylvestris* by Zahumenická et al. (2018) and *Spathiphyllum wallisii* (Eeckhaut et al. 2004). Tetraploid plants of *Anemone sylvestris*. were obtained in the medium concentrations. The highest concentration (15 µM/l) for the 12 weeks duration was toxic for all treated plants. The shorter time was needed for

induction of tetraploid plants (8 weeks) with increasing concentrations of oryzalin (Zahumenická et al. 2018). The lower concentrations were also successfully used by Ridwan & Witjaksono (2020) for inducing of polyploidy in *Moringa oleifera* where tetraploid plants were obtained in treatment of seeds for 1 day in 15 µM/l and 60 µM/l of oryzalin (Ridwan & Witjaksono 2020). *C. fragrans* is well adapting into *ex vitro* condition. The survival rate of transferred plants was 100 %. The tetraploids plants of *Anemone sylvestris* grown in the fields conditions had a 100 % survival rate in the next year, while the diploids had only 58 % (Zahumenická et al. 2018). Our obtained results are impossible to compare as there is no information about survival rates of transplanted plants of other species.

The chromosome number for *C. fragrans* is commonly reported as a 2n = 2x = 12 by many authors (Darlington 1929; Anderson & Sax 1936; Hunt 1986; Stuessy 1990; Saensouk & Saensouk 2020). In contrast, Nandikar et al. (2010) reported chromosome number as 2n = 2x = 18 in *C. fragrans*. This research confirms more frequent results obtained by Darlington (1929), Anderson & Sax (1936), Hunt (1986), Stuessy (1990) and Saensouk & Saensouk (2020) that *C. fragrans* chromosome number is 2n = 2x = 12.

Newly obtained genotypes have significant morphological differences. The number of leaves decreased in tetraploid plants of *C. fragrans* moreover the biomass increased in one genotype. Kaensaksiri et al. (2011) reported that despite the number of leaves of tetraploid plants *Centela asiatica* decreased. The fresh weight of tetraploid plants increased more than 77 %. The similar increase in biomass by polyploidization was reported by Gao et al. (1996, 2002) in *Salvia miltiorrhiza* and *Scutellaria baicalensis*.

Some genotypes of induced autotetraploid plants of *C. fragrans* exhibit the increase of leaves length. Similar results were obtained in *Thymus vulgaris* (Shmeit et al. 2020), *Salvia miltiorrhiza* (Gao et al. 1996) and *Rubus persicus* (Sabooni et al. 2022). Also induced tetraploid genotypes of *Moringa oleifera* generated larger leaflets (Ridwan & Witjaksono 2020).

Autotetraploid genotypes of *C. fragrans* generated significantly larger inflorescences with larger petals only one genotype generated flowers smaller than the control plant. The significant increase in flower size was also observed in *Anemone sylvestris* (Zahumenická et al. 2018) and *Chamaemelum nobile* (Tsuro et al. 2016). By

polyploidy of Rubus spp. the crown diameter increased by 10.29 mm in *Rubus caesiu*, in contrast *R. hirtus* exhibit decrease in flower size (Sabooni et al. 2022).

The variance in content of macro and micronutrients was detected. Potassium showed almost 100 % increase in all genotypes. The calcium content increased only in P2 genotype. The other genotypes have calcium content reduced in comparison with diploid plants. Protein, calcium and fat content significantly increased in induced tetraploid plants of *Moringa oleifera*. Protein content increased by 20 %, fat content increased up to 34 % and calcium content raised up to 20 % in tetraploid plants (Ridwan & Witjaksono 2020). The amount of chemical compound increased in lots of medicinal plants such as *Thymus vulgaris*, *Salvia miltiorrhiza* (Gao et al. 1996) and *Moringa oleifera* (Ridwan & Witjaksono 2020). The content of matrine and oxymatrine increased by polyploidy induction of Chinese medicine plant *Sophora tonkinensis.* The oxymatrine content raised by 107.1% in comparison with the control plant. About 0.087 mg/g was measured in radix by HPLC analysis (Wei et al. 2018). The biochemical compounds of *Callisia fragrans* needs to be a part of a further research.

In both used DNA based fingerprints method, control plants were separated from polyploidized ones. Polyploidization in its natural or *in vitro* conditions results in genome duplication and is an inevitable part of plants evolution and speciation. Different marker techniques were used to analysed polyploidized plants. Microsatellite based markers were used to characterize the fingerprints profiles of eupolyploidizated and aneuploidizated seedlings from seven diploid *Malus* populations (Considine et al. 2012). *Aronia melanocarpa* genetic structure in native polyploidy populations were analysed by RAPD (Randomly amplified polymorphic DNA (Hovmalm et al. 2004).

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 the variability of the retrotransposons insertion pattern was analysed in the diploid and tetraploid plants of *C. fragrans*. Here, iPBS analysis was applied for the first time for testing of stability of induced polyploid plants of *C. fragrans*. A completely monomorphic iPBS profile was obtained for the primer 1897 which generated 7 amplicons. The iPBS fingerprinting show a variability between all genotypes and the control plant, thus control plant was evidently different from all other genotypes. P1 and P2 showed the same profile by iPBS markers.

Up to now, many plant species were analysed by iPBS markers, such as *Liparis loeselii* (Belgorudova et al. 2012); *Saussurea esthonica* (Gailite & Rungis 2012) or *Prunus armeniaca* (Baránek et al. 2012). CDDP, similar to the iPBS, was applied firstly to analyse the *C. fragrans* genome. Three main branches were generated with CDDP profile, and the control plant generated fingerprint was separated clearly. Different marker techniques are actually used to analyse the polymorphism generated through the coding parts of the plant genomes, such as SCoT (Petrovičová et al. 2017), CDDP (Jiang & Zang 2018), TRAP (Vivodík et al. 2020) or PBA (Ravi et al. 2020).

There are lots of methods using DNA markers for detection of genetic changes such as inter simple sequence repeat (ISSR) (Štefúnová et al. 2016), simple sequence repeat (SSR) (Balážová et al. 2016), amplified polymorphic DNA (RAPD) (Kuťka-Hlozáková et al. 2016) and amplified fragment length polymorphism (AFLP) (Labajová et al., 2013). All of them were reported to be reliable (Kalendar et al. 2010; Kalendar & Schulman, 2014; Tiwari et al., 2013) and could be another alternative for analysis of genotype stability of *C. fragrans* in future analysis beside the iPBS and CDDP markers used in this study.

6. Conclusions

In vitro induction of polyploidy using oryzalin can successfully induce autotetraploids (2n = 4x = 24) in *C. fragrans*. The newly obtained genotypes are morphologically different from the diploid form. Also have higher nutrient content. DNA based analysis of polymorphism were performed by iPBS and CDDP markers, which were applied for the first time for *C. fragrans*. Newly acquired genotypes showing different traits extend the genetic variability of *C. fragrans* and prove that *in vitro* oryzalin-induced polyploidy can be a valuable breeding strategy for production improved clones with new features of *C. fragrans.* The presented results are obtained after two years of cultivation of *C. fragrans* plants. It is recommended obtained genotypes to be part of further observation to approve stability of obtained traits.

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Figures:

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Figure 3: Trilling J. 2022. *Tradescantia zebrina* Bosse https://www.inaturalist.org/observations/106646900 (accessed April 20, 2022).

Figure 4: Ramirez G. 2021. *Tradescantia pallida* (Rose) D.R.Hunt https://www.inaturalist.org/observations/70040051 (accessed April 20, 2022).

Appendices

List of the Appendices:

Appendix 1: The composition of the MS medium

Source: Murashige and Skoog (1962)

Stock solutions for 1 litre of distilled water		Batch size to 1 litre of stock solution	On the 1 litre to measure (pH 5,7)
A	NH_4NO_3	16.5 g	100 ml
	KNO_3	19 g	
	$CaCl_2$	3.3 g	
	$MgSO_4 \times 7H_2O$	3.7 g	
	KH_2PO_4	1.7 g	
B	H_3BO_3	620 mg	10 ml
	$MnSO_4 \times 4H_2O$ (H_2O)	2.23 g (1.69 g)	
	$ZnSO_4 \times 4H_2O$ ($7H_2O$)	860 mg (1.06 g)	
C	KI	83 mg	10 ml
	$Na_2MoO_4 \times 4H_2O$	25 mg	
D	$CuSO_4 \times 5H_2O$	2.5 mg	10 ml
	$CoCl_2 \times 6H_2O$	2.5 mg	
E	Na_2EDTA	3.72 mg	
	$FeSO_4 \times 7H_2O$	2.78 mg	
V	nicotin acid	50 mg	10 ml
	pyridoxin (B6)	50 mg	
	thiamin (B1)	10 mg	
	glycin (amino acid)	200 mg	

Direct batch size to the medium:

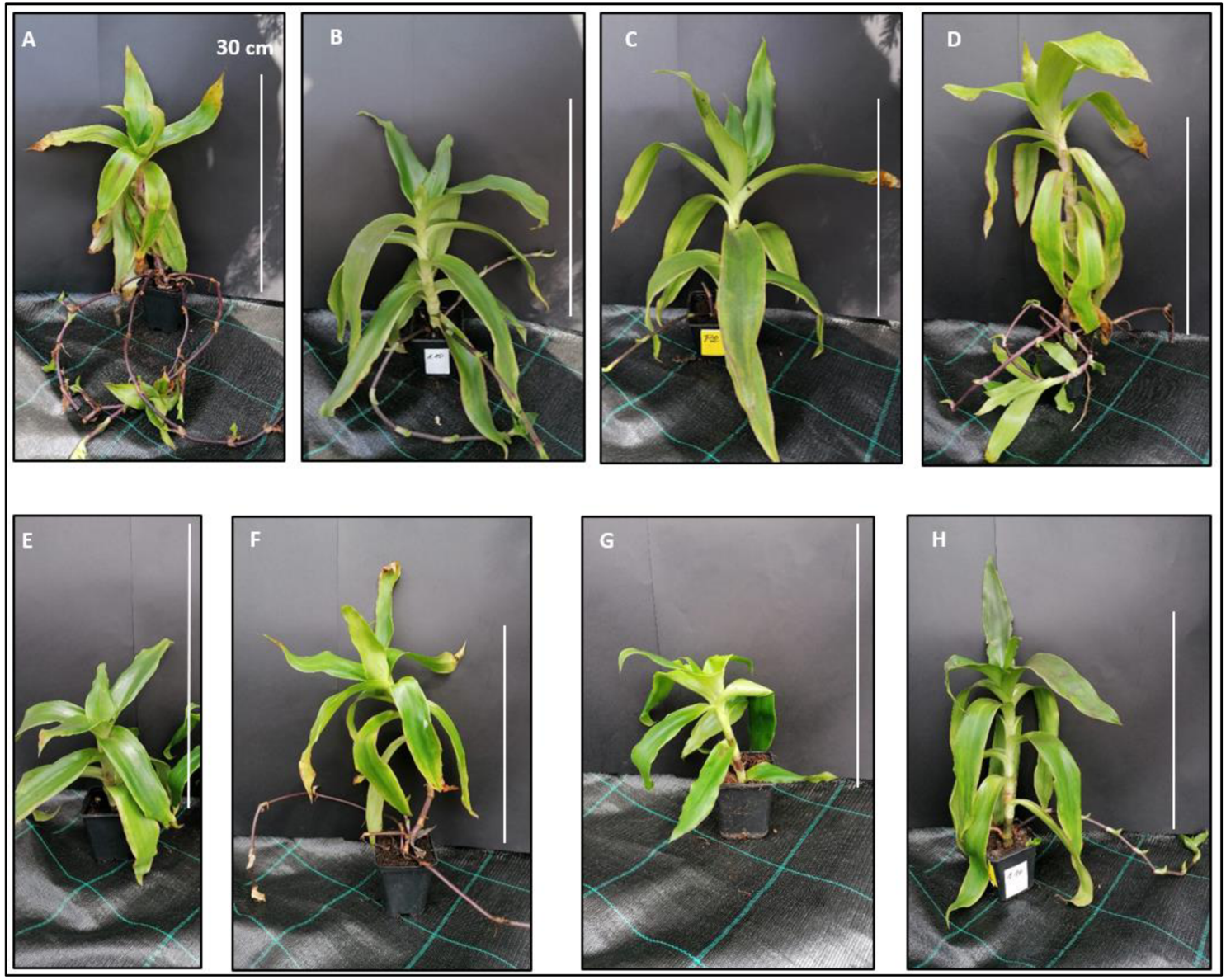
Mioinositol 0.1 g

Sucrose 30 g

Agar 8 g

Appendix 2: Growth of *C. fragrans* 4 months old plants: **A.** Autotetraploid genotype P1; **B.** Autotetraploid genotype P2; **C.** Autotetraploid genotype P3; **D.** Autotetraploid genotype P4; **E.** Autotetraploid genotype P5; **F.** Control genotype; **G.** Mixoploid genotype M1; **H.** Mixoploid genotype M2

Appendix 3: 5 months old plants of *C. fragrans*: **A.** Control plant; **B.** Autotetraploid P1; **C.** Autotetraploid P2; **D.** Autotetraploid P3; **E.** Autotetraploid P4; **F.** Autotetraploid P5; **G.** Mixoploid M1; **H**. Mixoploid M2



Appendix 4: Comparisons between genotypes of *C. fragrans*: **A.** Control genotype; **B**. Autotetraploid genotype P1; **C.** Autotetraploid genotype P2; **D.** Autotetraploid genotype P3; **E.** Autotetraploid genotype P4; **F.** Autotetraploid genotype P5; **G.** Mixoploid genotype M1; **H**. Mixoploid genotype M2

Appendix 5: Morphological variation in *C. fragrans* flowers compared to control: **A** Control genotype; **B.** Autotetraploid genotype P1; **C.** Autotetraploid genotype P3; **D.** Autotetraploid genotype P4; **E.** Autotetraploid genotype P5