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AgriSciences**

***in vitro* induction polyploidy of *Lycianthes
asarifolia* (Kunth & Bouché) Bitter**

BACHELOR'S THESIS

Prague 2020

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Declaration

I hereby declare that I have done this thesis entitled *in vitro* induction polyploidy of *Lycianthes asarifolia* (Kunth & Bouché) Bitter 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 date:

.....

Vera Zakamennaia

Acknowledgements

I would like to thank my bachelor thesis supervisor prof. Ing. Eloy Fernandez Cusimamani for professional advice and guidance in preparing a bachelor's thesis.

I would also like to thank the Faculty of Tropical Agriculture, which allowed me to work in the Laboratory of Plant Explants.

I would also like to thank my classmate Kateřina Beranová for advices and support, also team of doctoral students in the Laboratory of Plant Explants with the help of an experiment.

Finally, I would like to thank my mother for her unconditional love and encouragement.

Abstract

Lycianthes asarifolia (Kunth & Bouché) Bitter belongs to Solanaceae family. It is a fruiting plant origin from Latin America.

The aim of this bachelor thesis was to obtain tetraploid plants ($2n = 4x = 48$) from original diploid ($2n = 2x = 24$), by *in vitro* induced polyploidy.

For polyploidisation were used nodal segments that were exposed to the anti-mitotic agent oryzalin at concentrations of 20 μM , 40 μM , and 80 μM for 24 and 48 hours. These nodal segments were cultured on Murashige-Skoog (1962) medium without growth regulators. The ploidy level was detected by flow cytometry.

In total were obtained 9 mixoploid plants, the most of after oryzalin treatment in 40 μM for 48 hours, also at 40 μM for 24 hours, and in concentration 80 μM for 24 and 48 hours. Tetraploid plants were not obtained. Whether a higher concentration of oryzalin or the use of another anti-mitotic agent is required for the induction of polyploidy in *L. asarifolia*.

Key words: *Lycianthes asarifolia*, Solanaceae, *in vitro*, Flow cytometry, fruit plants, micropropagation, oryzalin, polyploidisation

Abstrakt

Lycianthes asarifolia (Kunth & Bouché) Bitter je ovocný druh, patřící do čeledi Solanaceae, původem z Latinské Ameriky.

Cílem této bakalářské práce je získat tetraploidní rostliny ($4n = 4x = 48$) z původních diploidních rostlin ($2n = 2x = 24$) *in vitro* indukovanou polyploidii.

Polyploidie byla indukována u nodálních segmentu pěstovaných na MS médiu (Murashige-Skoog, 1962) bez přídavku regulátorů růstu a anti-mitotického činidla oryzalinu v koncentracích 20 μM , 40 μM a 80 μM po dobu 24 a 48 hodin. Úroveň ploidie u ovlivněných rostlin byla stanovena pomocí flow cytometrie.

Celkově bylo získáno 9 mixoploidních rostlin. Většina mixoploidů byla získána v koncentraci 40 μM po dobu 48 hodin, také v koncentraci 40 μM po dobu 24 hodiny a v koncentraci 80 μM po dobu 24 a 48 hodin. Tetraploidní rostliny nebyly získány. Zdá se, že pro indukci polyploidu u *L. asarifolia* je zapotřebí použití vyšší koncentrace oryzalinu nebo jiného antimitotického činidla

Klíčová slova: *Lycianthes asarifolia*, Solanaceae, *in vitro*, flow cytometrie, ovocné rostliny, mikropropagace, oryzalin, polyploidizace

Contents

1. Introduction	1
2. Literature Review	2
2.1. Solanaceae family	2
2.2. Taxonomy classification of <i>Lycianthes asarifolia</i> (Kunth & Bouché) Bitter .	4
2.3. Morphology of <i>Lycianthes asarifolia</i> Bitter.....	5
2.4. Distribution and ecology of <i>Lycianthes asarifolia</i>	8
2.5. Using.....	8
2.6. Micropropagation	8
2.6.1. Micropropagation of fruit plants.....	9
2.6.2. Micropropagation of <i>Solanaceae</i> family.....	9
2.7. Plant breeding	10
2.7.1. Polyploidisation	11
2.7.1.1. Mitotic polyploidisation	12
2.7.1.2. Meiotic polyploidisation	12
2.7.1.3. Anti-mitotic agents.....	13
2.7.1.4. Ploidy level detection	16
3. Aims of work.....	17
4. Materials and methods	18
4.1. Plant material	18
4.2. Derivation of aseptic culture.....	18
4.3. Polyploidy induction.....	19
4.4. Determination of ploidy level.....	19
5. Results	21
5.1. Morphology evaluation.....	23
6. Discussion	26
7. Conclusion.....	28
8. References	29

List of figures

Figure 1:	Fruits of <i>Lycianthes asarifolia</i> .	6
Figure 2:	Flower of <i>Lycianthes asarifolia</i>	6
Figure 3:	<i>Lycianthes asarifolia</i>	7
Figure 4:	Chemical structure depiction of oryzalin	15
Figure 5:	Chemical structure depiction of colchicine	15
Figure 6:	Schematic diagram of flow cytometer	16
Figure 7:	Histogram of control plant, peak on channel 200 correspond to the nuclei of the control plant	21
Figure 8:	Histogram of the mixoploid plant affected by 40 concentration for 24 hours.	22
Figure 9:	Leaf of control plant	25
Figure 10:	Plant treated in 40 μ M for 24 hours.	25
Figure 11:	Plant treated in 80 μ M for 24 hours.	25
Figure 12:	Plant treated in 40 μ M for 48 hours.	25

List of the abbreviations used in the thesis

MS Medium	Medium Murashige - Skoog (1962)
DAPI	4',6-diamidino-2-phenylIndole
BA	Benzyl adenine
ABA	Indole-3-butyric acid
TWEEN 2	Polysorbate
DMSO	Dimethyl sulfoxid

1. Introduction

Genus *Lycianthes* (Dunal) Hasl contain around 150- 200 species (Silva 2018). This plant belongs to *Solanaceae* family with a cradle of origin in Latin America (Jagatheeswari 2014). *Lycianthes* species are difficult to collect due to their occurrence in restricted environments or because they are in possible extinction on the grounds of the destruction of their habitats. However, a few species are widely spread and have economical importance since they can be cultivated as food. Specie *Lycianthes asarifolia* (Kunth & Bouché) Bitter is reported to be edible due to sweet taste of its fruits (Silva 2018).

Creeping green herb with solitary 2-13 cm long and 2,5-6 cm wide cordate shape, glabrous leaves. Sympodial units plurifoliate. Inflorescence is attached directly by the base, single flowered. Flower is solitary as well, perfectly pentamerous. White corolla is 0,6—1.1 long and 1,2—2,2 cm wide. Pedicel is 5-8 cm long, separated by nodes covered with sparse hairs. Fruits are globose, orange berries, 1,4-1,7 cm in diameter with brown seeds inside (Silva 2018).

Solanaceae family's genera such as potato, tomato or peppers are spread much outside Latin America, and are one of the most important market crops (Jagatheeswari 2014). *Lycianthes asarifolia* plant is used for consumption and jams making, also mushed leaf buds used external for boils treatment (Molina & Javier 2016; Bourdy et al. 2000). Although biochemistry studies of *L. asarifolia* are requested.

The *Lycianthes* genus is example of poor collected and represented in herbaria plant. It is a relevant aspect from conservation point of view. Various breeding methods are used in plant breeding programs, one of which is somatic induced polyploidisation *in vitro*. Using this method, it is possible to improve the ornamental potential of plants and increase the constituents of medicinal plants (Roughani et al. 2018).

The presented bachelor thesis deals with the issue of induced polyploidisation in *in vitro* cultures. Using polyploidisation, new genotypes with new morphological properties can be obtained.

2. Literature Review

Genus *Lycianthes* Hassl. L is dicotyledonous plant member of *Solanaceae* family, which approximately contains 3000 species (Silva 2018; Jagatheeswari 2014).

Lycianthes asarifolia (Kunth & Bouché) Bitter is the most frequently used scientific accepted name of this species but could be found under *Solanum asarifolium* Kunth & Bouché as well (Silva 2018). *L. asarifolia* is also known by common names „ginger leaf” in english, and as Bolivian „Motojobobo” (Mostacedo et al. 1999). *Lycianthes asarifolia* has both Old and New World representatives, but its centre of distribution and the majority of taxa are found in the New World from Venezuela to Paraguay and the Brazilian Amazon, also can be found in North America as invasive species (Tropicos 2020).

It is a Creeping grass, whose roots come out from the knots of the stem. Heart-shaped, rounded leaves, dark green. Solitary flower, which sprouts from the leaf axils, white with the yellow anthers in the centre. Berry fruit, spherical-elongated, orange when ripe, with fleshy and firm pulp, sweet, acidulated, pleasant flavour, with many small seeds. Flowered specimens were observed through the year, and frutified specimens were registered from May to December (Molina & Javier 2016). Usually grows among grasses of low size that provide some shade (Silva 2018).

Lycianthes asarifolia fruits could be consummated bare, but it is customary to make jams. The flavour is very refined and lends itself to making liqueurs, jellies and sweets (Williams 1993; Samuels 2015). It also has ornamental applications such as ground covers (Molina & Javier 2016).

According to The National Center for Biotechnology Information (NCBI) *Lycianthes asarifolia* chromosome number is $2n=24$ (NBCI 2020; Moscone 1992).

2.1. Solanaceae family

The family Solanaceae is a perennial and nightshade plants includes approximately 3000 species that are classified in approximately 90 genera. Members of this family includes herbs, shrubs trees, also medicinal and ornamental plants

(Jagatheeswari 2014). Many species contain steroidal glykoalkaloids biosynthesised for the protection of the plants, some are with highly toxicity (solanin, atropine, nicotine) , but plenty of them are considerable as food and very marketable such as tomatoes (*Solanum lycopersicum*), potatoes (*Solanum tuberosum*), eggplant (*Solanum melongena*) or chilli pepper (*Capsicum annuum*). And some species are considerable as drugs tobacco (*Nicotiana tabacum*), belladonna (*Atropa belladonna*), the poisonous jimsonweed (*Datura stramonium*). Solanaceae family ranges throughout the Nootropics and Southeast Asia has a great diversity of habitats, morphology and ecology (Jagatheeswari 2014).

The family is worldwide distributed and presented and successfully cultivated on all continents except Antarctica.

Blades of leaves can be simple or compound, with bases ranging from cordate to tapering.

Leaf could be glabrous or has simple hair taking assorted forms. Spines often occur on the calyx, leaves, and stems and can be straight or curved, broad-based or needle-thin.

This morphology marks are important for taxonomy diagnostic in *Solanum* group (Jagatheeswari 2014).

Flowers generally are actinomorphic: corolla 5-lobed calyx, and a 2-carpellate superior ovary, and 5 stamens. Anthers open by terminal pores, with age pores elongate into slits in many species. Usually very short filaments, and in many species the anthers are connate around the style into a tight cone (Jagatheeswari 2014).

Fruit morphology can show great variation across the genus: bright juicy berries (*Solanum dulcamara*) or soft greenish berries (*Solanum mite*). The generalised fruit in *Solanum* is a 2-carpellate berry or a capsule.

Solanaceae family is known for various alkaloids form content with intense physiological effect on animals. Those secondary metabolites are found in plants such as henbane (*Hyoscyamus albus*), belladonna (*Atropa belladonna*), jimson weed (*Datura stramonium*), mandrake (*Mandragora autumnalis*) and others. The most known types of alkaloids are nicotine and capsaicin (Ciechomska & Woźniakiewicz 2019).

Nicotine, found in the tobacco plant (*Nicotiana tabacum*), is addictive central nervous system stimulant. Nicotine is an organic alkaloid that is a major component of cigarettes and is used therapeutically to help with smoking stoppage (PubChem Database 2020).

Capsaicin extracted from chilli pepper (*Capsicum annuum*) and has analgesic properties. This irritant and acute toxic agent aids in controlling peripheral nerve pain if used topically (PubChem Database 2020).

2.2. Taxonomy classification of *Lycianthes asarifolia* (Kunth & Bouché) Bitter

According to Global Biodiversity Information Facility first record of *Lycianthes asarifolia* (Kunth & Bouché) Bitter was in 1821 March in Brazil, 77 years later, October 1898, also in Brazil, this species was recorded under *Solanum chodatianum* Huber scientific name.

Other confirmed scientific names include: *Solanum violifolium* var. *asarifolium* (Kunth & Bouché) Hassl (Jørgensen et al 2014), *Solanum violifolium* f. *lilaciniflorum* Hassl, *Solanum violifolium* var. *majus* Dunal (Hokche 2008)

In spanish language commonly known as „Barba de Tigre”, „Childa” or „Motojobobo”, in english as „Ginger leaf” (De Rojas & D’Arcy 1997). Most number of found plants were found in Bolivia, its South-East region specifically.

Studies of karyotype of *Lycianthes* species has shown that chromosome number $2n=24$, it is a diploid organism (NBCI 2020; Moscone 1992).

Taxonomy of *Lycianthes asarifolia*:

- Kingdom	<i>Plantae</i>
- Subkingdom	<i>Viridiplantae</i>
- Infra kingdom	<i>Streptophyta</i>
- Superdivision	<i>Embryophyta</i>
- Division	<i>Tracheophyta</i>
- Subdivision	<i>Spermatophytina</i>
- Class	<i>Magnoliopsida</i>
- Superorder	<i>Asteranae</i>
- Order	<i>Solanales</i>
- Family	<i>Solanaceae</i>
- Genus	<i>Lycianthes (Dunal) Hassl.</i>
- Species	<i>Lycianthes asarifolia (Kunth & Bouché) Bitter</i>

Source : Integrated Taxonomic Information System (ITIS) 2020

2.3. Morphology of *Lycianthes asarifolia* Bitter

Lycianthes asarifolia (Fig 3) is procumbent creeping herb with slender stems and stolons, and delicately pubescent. Leaves are from middle to dark green colour, with glossy abaxial surface. Slightly puberulous or glabrous. Leaves could be cordate or sagitate shaped at base. Size of the leaf is approximately 2 - 6,5 x 2,5 - 6 cm. Grow solitary on 2 - 13 cm long canaliculate pedicel. Inflorescence is solitary as well, one-flowered with trichomes similar to those on the branches. Flowers are perfectly pentamerous growing on 5 - 8 cm long curved at the apex pedicel. Flowers (Fig 2) having stamens and pistils are in the same flower — monoclinal. Calyx is bell-shaped, 2,5 - 4 cm long, not clearly visible usually is evidence of dry plant. Corolla is white, wheel-shaped, with the limbs of petals being at right angle to the tube, size is 0,6 - 1,1 x 1,2 - 2,2 cm, lobes is 0,6 - 1,2 x 0,3 - 0,6 cm. 5- statements. equal androecium with filament 1,3 - 1,5 mm long, and 2,5 - 3,5 mm long, anthers. Gynoecium is glabrous, 1,5 - 2,0 mm diameter. Berries are spherical-elongated, orange when ripe, 1,4—1,7 cm diameter (Silva 2018). Pulp of the fruit is fleshy with many small seeds (Fig 1.) (Molina & Javier 2016).



Fig 1. Fruits of *L.asarifolia*.



Fig 2. Flower of *Lycianthes asarifolia*.

Autor: Molina C, Javier D.



Fig. 3 *Lycianthes asarifolia*

Autor: A. Parada. Vallegrande - Bolivia

2.4. Distribution and ecology of *Lycianthes asarifolia*

Lycianthes asarifolia presents a wide range of distribution, occurring from Venezuela to Paraguay and the Brazilian Amazon, also can be found in the northeast region as an invasive species (Silva 2018). *L. asarifolia* grow near the sea, in lowlands in eastern South, so called Yungas the zone between the Andean highlands and the eastern forests. The Yungas belong to the Neotropic ecozone; the climate is warm and humid. Latitude of Yungas is laying between 500 and 2,300 m above the sea. *L. asarifolia* could be found near moist places at 50 - 1000 m latitude (Zygodlo et al. 1994). It occurs in disturbed areas, along roads or on forest edges (Silva 2018). Grows among grasses of low size that provide some shade, if there is dense shade of trees it becomes dominant (Molina & Javier 2016). Flowered specimens were observed through the year, and frutified specimens were registered from May to December, if, there is humidity (Silva 2018; Molina & Javier 2016).

2.5. Using

Fruits are eatable bare and could be eaten as jams. Fruiting and flowering all through the year. The motojobobo flavour is very refined and lends itself to making liqueurs, jellies and sweets (Samuels 2015). Could be cultivated as ornamental ground covers (Molina & Javier 2016)

Also known some medical external usage of mushed leaves of *Lycianthes asarifolia*, although further biochemical compound studies are required (Bourdy et al. 1999).

2.6. Micropropagation

Micropropagation is the artificial process of producing plants vegetatively through tissue culture or cell culture techniques. In this artificial process of propagation, plants are produced *in vitro* by asexual means of reproduction or by vegetative propagation.

Plants can be produced both asexually by vegetative parts multiplication or sexually - seed production. One of the means of asexual reproduction is by multiplying genetic replicas of plants that are referred to as clonal propagation wherein plants can be populated from a single individual through asexual means of reproduction.

For the *in vivo* propagation of specific plants, asexual reproduction via multiplication of vegetative parts is the only resort since they do not generate functional seeds as seen in figs, grapes, bananas etc. (Bhatia & Sharma 2015).

2.6.1. Micropropagation of fruit plants

Fruit plants have a giant market value and play an important role in the human diet. Usually export of fruits are important part of income of tropic countries. Fruits are known to contain several health-promoting components, such as vitamins, essential minerals, antioxidants even prebiotic (Hanke & Flachowsky 2010). Commercial production of fruit plants is widespread throughout the world. Also, propagation of any other plant type is necessary to create a new variety of well know plant and maintain diversity. As usually propagation is maintained by vegetative multiplying, this method keeps needed properties of mother plant trough new offspring's and increase the production of seedlings with highly superior merits. For propagation many developed agricultural technique are used such as grafting or budding. Also used biotechnologies for propagation of small plant tissues *in vitro*. Micropropagation *in vitro* is used for rapid propagating material production, photogenic removal, germs protection, second metabolites production or even genetic manipulation (Iliev & Gajdoov 2010).

2.6.2. Micropropagation of *Solanaceae* family

Due to the fact, that most of the *Solanaceae* family species are important market crops, micropropagation of chosen plant is properly studied (Gebhardt 2016). The first ever plant what was examined on Murashige and Skoog, newly invented media, was the tobacco plant (*Nicotiana tabacum L., var. Wisconsin 38*) (Murashige & Skoog 1962).

One of the most favoured Solanaceae family plant to *in vitro* micropropagation and improvement is tomato (*Lycopersicon esculentum* Mill.). The main problem for breeders is to transfer the higher regenerative ability of wild varieties to cultivated tomatoes and to breed disease-resistant crops (Bhatia et al. 2004). As explant tissue are used cotyledon and hypocotyl segments young tomato seedlings (Saeed & Naseem 2019).

Another *Solanaceae* plant to study is potato (*Solanum tuberosum* L.). For asexual micropropagation are used sprouts and nodal explants. The main goal is breed viruse resistant cultivar or produce micro tubers for improved storage, transportation and bulk production in any season (Koleva Gudeva et al. 2012).

Micropropagation method for the *Lycianthes* (Dunal) Hassl. is not very widespread and few authors have dealt with the issue.

Lycianthes bigeminata Bitter is an important medicinal herb, used for curing ulcer. Padmavathy & Paulsamy (2007) had an attempt to know the response on various concentrations of hormones for further size increase experiments of plant.

In studies by Niño & Correa (2006), *Lycianthes synanthera* (Send.) Bitter was one of the plants with the strongest antibacterial activity against microorganism.

Lycianthes asarifolia could be a potentially valuable agricultural crop for Latin America market, or even potential medicinal herb, so further biochemical studies are required.

2.7. Plant breeding

Plant breeding is crucial factor for plant properties improvement such as disease or pest resistance, environmental stress tolerance, adaptation. The higher quality and diversity gain by different breeding methods. Plant breeding can help with plant development for specific purposes and improve production of important crops.

Breeding can be performed by variety of methods, from selection of plants by its morphology properties to complex molecular techniques. The process of breeding new varieties involves crossing and subsequent selection. The crossing process can be performed by remote hybridisation, by increasing the number of chromosomes by

polyploidisation, or by inducing mutations by ionising radiation or chemical agents (Graman 1998).

2.7.1. Polyploidisation

Polyploidy also known as WGD (whole - genome duplication) the condition in which a normally diploid cell or organism acquires one or more additional sets of chromosomes (Graman 1998). Can be found in wide spectrum of eucaryotes. Shift in polyploidy has direct morphology impact on size of cell, mitosis, cell cycles or indirect impact on adaption potential of species (Levin 1975).

Ploidy is the common definition for variety of chromosomes number in nucleus of a cell. In somatic cells chromosomes exist in pairs - this condition is called diploidy (Graman 1998).

Produced during meiosis germ cells, contain only half the somatic number of chromosomes - this condition is haploidy (Fincham 2001).

Cells which have three, four or more times the number of chromosomes in haploid cells are polyploids. Polyploidy occurs in plant as a result of chromosomes duplication without cytoplasm division or could be result of union of diploid gametes. Infertility is common for polyploid plants (Graman 1998).

Frequently, in homologous pair of two chromosomes one more chromosome could occur or disappear. This aberration is aneuploidy (Endo et al. 2019).

Polyploidy occurs naturally in all plant species, and it is a major evolutionary force. Polyploid organisms often present increased vigor and regularly outperform diploid relatives in many aspects. This natural plant ability has been studied and improved in laboratory environment for past century by many plant breeders. Polyploidy plant provides improved product quality and abiotic and biotic stress tolerance. Polyploid can be used as a platform between breeding two species with different ploidy levels (Sattler et al. 2015)

For artificial induction of polyploidy used irritation by different agents such as:

- physical - temperature, ionising radiation, x-ray, infections
- mechanical - centrifuge, material damage
- chemical - colchicine, chloral hydrate, chloroform, nitrous oxide, oryzalin.

Chromosome duplication in plants has been traditionally accomplished by the use of colchicine (Portela et al. 2005). Colchicine is generally used as water solutions at various concentrations, in glycerine or agar medium, but this chemical usually led to plant sterility after, and can be highly toxic for humans. Recently mostly used different herbicides such as oryzalin or trifluralin or any other chemical agents (Salma et al 2017). Concentration and application time chosen according to plants part and its ability to recover.

For polyploid plants productions two mechanisms are used - mitotic polyploidisation or meiotic polyploidisation (Hrdličková 2014).

Polyploids can be induced by two mechanisms: mitotic polyploidisation, which is based on the doubling of chromosomes in somatic tissues; and meiotic polyploidisation, which generates $2n$ gametes (Dhooghe et al. 2011).

2.7.1.1. Mitotic polyploidisation

Mitotic polyploidisation, which is asexual, based on doubling chromosomes in somatic tissue by interfering with the plant's cell cycle and inhibiting the dividing spindle activity (Hrdličková 2014). First application of mitotic polyploidisation *ex vitro* was recorded by Blakeslee and Avery in 1930s. Then in year 1966 first mitotic polyploidisation *in vitro* attempting by Murashige and Nakano on *Nicotiana tabacum* L. (Murashige & Nakano 1967)

The most commonly used anti-mitotic agent is colchicine, oryzalin or trifluralin (Hrdličková 2014)

2.7.1.2. Meiotic polyploidisation

Sexual meiotic polyploidisation results in the production of unreduced gametes ($2n$), diploids.

After two polyploid gametes are met the form a polyploid embryo, in case of meeting diploid gamete with haploid aneuploid is occurs. New polyploid off springs φraise depending in the chosen genotype and environmental conditions. Consequently meiotic polyploidisation provides more information on genetic factor that affects the frequency of diploid gamete production (Carputo et al. 2003).

2.7.1.3. Anti-mitotic agents

Anti-mitotic agents could be synthetic or even natural compounds with a capability of cell division distortion and mitosis inhibition, could be applied in both ways: *in vitro* or *ex vitro* (Yemets & Blume 2008; Schmeit 2015).

With colchicine isolation from the seeds and bulbs of the wild meadow saffron *Colchicum autumnale L.*, its usage for polyploidisation has started (Takamura et al. 2002; Yemets & Blume 2008).

Colchicine (Fig 5) causes side effects such as sterility, abnormal growth, rearrangements, gene mutation or chromosome loose (Luckett 1989). Also, colchicine is very toxic to humans due to its high affinity to microtubules of animal cells (Takamura et al. 2002; Yemets & Blume 2008).

Due to colchicine inability to binds to plant tubulins properly, it must be used in relatively high concentrations, and soon, required to find alternative cell cycle inhibitor agent.

In 1990s, then herbicides started to use as anti-microtubular agents. It has been found that the herbicide oryzalin binds specifically to plant tubulins and can be used in lower concentrations. In comparison oryzalin is more stable in polyploid induction and explants have a higher survival rate after treatment (Ascough et al. 2008; Sakhanokho et al. 2009).

Oryzalin (Fig 4) is dinitroaniline (dinitroaniline (4-(Dipropylamino)-3,5 dinitrobenzenesulfonamide)) agent developed as a herbicide, used as an alternative to colchicine, intended for mitosis disruption by inhibiting the formation of microtubules in meristematic cells of seeding plants (PubChem Database 2020 ; Tuyl & Meijer 1992). Affects physiological growth processes associated with seed germination (Hartley & Kidd 1987)

Oryzalin is less toxic alternative to colchicine and needed in much lower concentration (Tuyl & Meijer 1992). Many studies comparing efficiency of colchicine to oryzalin point to that survivability, after-treatment regeneration and played level is higher in favour of last one (Ascough et al.2008).

Oryzalin has widely commercial use, controlling annual grasses, broadleaf weeds, woody shrubs and vines in grapes, berries and orchard crops. Formulation types include granular, wettable powder, water dispersible granules, emulsifiable concentrate, flowable concentrate and liquid.

Studies and animal testing have not shown any special toxic properties, although could be a carcinogenic factor for rats and classified as possible human carcinogen. Contact with skin or inhalation can cause slight eye irritation.

Biodegrade half-life of oryzalin is approximately two months, with 20% possibility of leach.

Oryzalin can be moderately toxic to fresh water and fish and as an herbicide rich non-target plant (Environmental Protection Agency 1994)

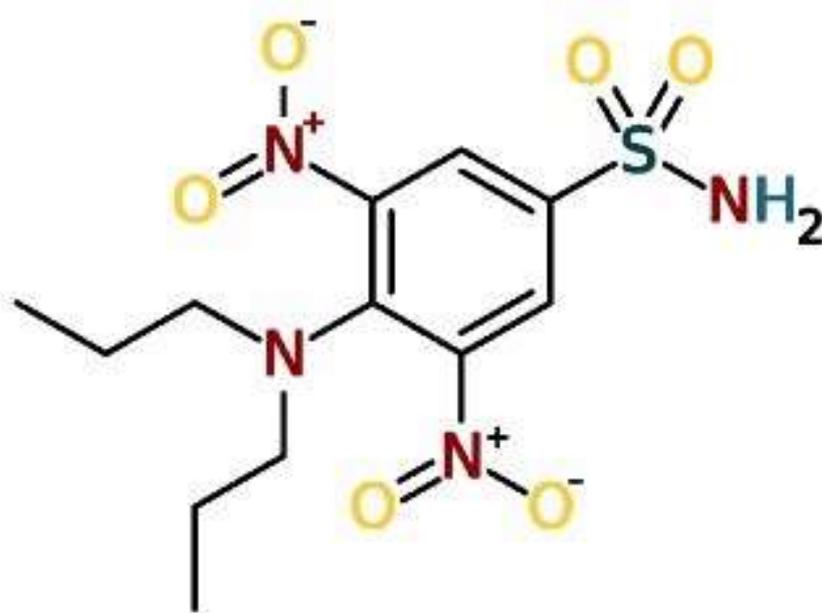


Fig 4. Chemical structure depiction of oryzalin

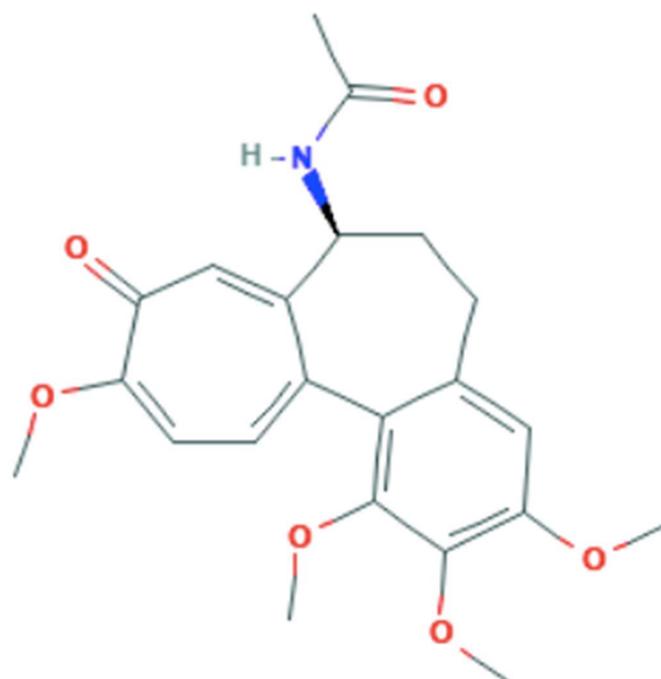


Fig 5. Chemical structure depiction of colchicine

Autor: Pub Chem 2020

2.7.1.4. Ploidy level detection

Exist plenty of chromosome number confirmation methods, such as chromosome counts, biochemical analysis and morphological observations, or advanced technical methods such as flow-cytometry. Polyploids, could be detected by morphological criteria as well (Dar & Rehman 2017).

Flow Cytometry is usual used for plants as cell cycle analysis and the nuclear DNA estimation method (Dar & Rehman 2017). This technology measures and analyses multiply physical properties of cell dyed with a DNA-binding fluorescent tint and passing through a stream of laser in fluid.

This method requires isolated and soaked in buffer solution nuclei from leaf of control sample and presumed polyploid sample. Then made solution passed through series of light, lenses, mirrors and amplifiers. Amplifiers detect amount of light and convert it into a digital signal. Intensity of fluorescence show the DNA content and can be two samples could be compared. As a polyploid can be considered a cell containing three or more sets of chromosomes (Leus 2009; Roughani & Miri 2018).

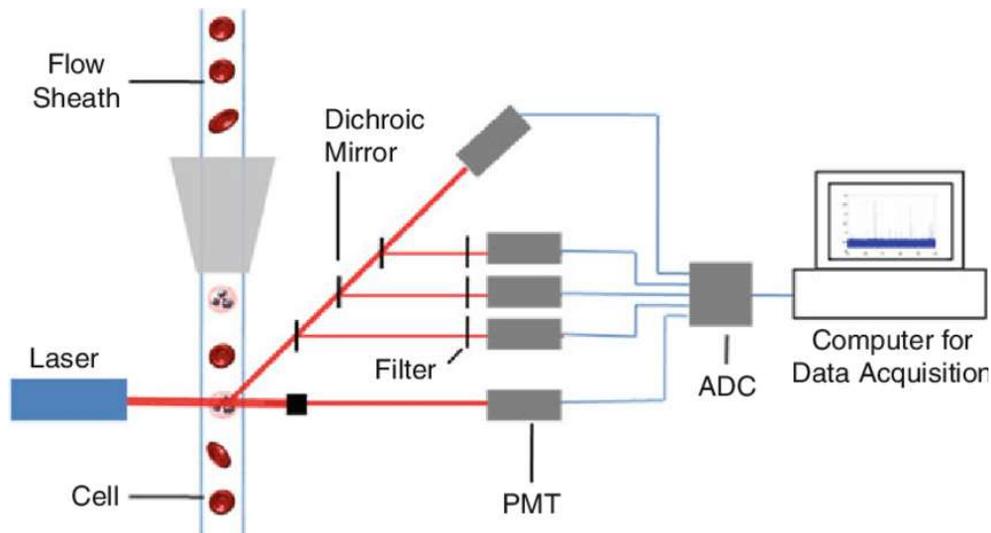


Fig 6. Schematic diagram of flow cytometer

Autor: Rongrong Liu 2017

3. Aims of work

The main aim of work was to maintain tetraploid plants with $2n=4x=48$ from diploid plants $2n=2x=24$, with *in vitro* induced polyploidy of *Lycianthes asarifolia* (Kunth & Bouché) Bitter.

The aim of work is established by two followed hypotheses:

- H1: oryzalin is effective Anti-mitotic agent for polyploidisation *L. asarifolia*
- H2: it is possible to maintain new genotype of Anti-mitotic agents *L. asarifolia* within *in vitro* culture with different morphology and biochemistry characteristic

4. Materials and methods

The polyploidisation process was performed in the Laboratory of Plant Explants at the Department of Tropical Crops and Agroforestry, Czech University of Life Sciences in Prague in 2019 to 2020.

4.1. Plant material

Fruits of *L. asarifolia* have been delivered from Bolivia, from the natural habitat for this genus. Seed was manually collected from fruit and dried on open air for a week. Then seed were used for further propagation on MS media in laboratory conditions

For proliferation and multiplication was used a Murashige and Skoog 1962 medium (via attachment), without any special treatment. The pH of the medium was adjusted to 5.8 using KOH before autoclaving. The 25 ml of the molten medium was distributed into conical flasks and covered with double layer of tinfoil then sterilised in autoclave 121 °C for 20 minutes.

Dry seeds were sterilised in 70% ethanol solution for 1 min then three times washed in sterile distilled water and then soaked in 1% sodium hypochlorite solution (NaClO) for 25 min for disinfection. Before distributing seed on 25 ml of aseptic MS medium they were rinsed with sterile distilled water three times. Seeds were placed in 100 ml Erlenmeyer flasks, 4 seeds per flask. Flasks were wrapped around in tinfoil to induce faster sprouting and leaved in cultivation room for two weeks, then tinfoil was removed, and young shoots were leaved there for 3 weeks more.

4.2. Derivation of aseptic culture

Before experiment initiation clones of one best grown plant of *L. asarifolia* is requested. A sufficient number of plants were propagated using nodal segments. The ability of ginger leaf to rapid grow, allowed to cultivate new plants and shoots forming fast enough without any additional treatment with grow regulators. Plants were grown on

pure MS medium in 100 ml Erlenmeyer flasks. After 3 weeks of cultivation up to 3 nodes were formed on one plant.

The plants were grown in a culture room with a light intensity of 3000 lx, a day temperature of 25 ° C / 16 h and a night temperature of 20 ° C / 8 h.

A sufficient amount (about 100) of plant material was cloned from the mother plant after 4 months of cultivation.

4.3. Polyploidy induction

Polyploidisation was performed in nodal segments with anti-mitotic agent oryzalin. Stock solution of oryzalin was mixed with 720 ml of sterilised distilled water for the preparing the working solution about 20 µM, 40 µM, and 80 µM concentrations for the experiment. Nodes of plant were placed in twelve 250 ml beakers with 50 ml of pure MS medium without any growth regulators addition. After 2 days of regeneration process, before fresh offshoots were designed, these segments were perfused in 60 ml of oryzalin of three different concentration (20 µM, 40 µM, 80 µM) for two time of treatment 24 and 48 hours. About 10 shoots per one beaker, in total 180 nodes, 30 nodes per every concentration and time interval.

After soaking in chemical solution nodal segments were rinsed three times in sterile distilled water and placed in new MS in beakers for further regeneration and growth.

After one the week shoots were placed in individual Erlenmeyer conical flask for convenient nodes and leaves growth. And had been systematically replaced on fresh medium every two weeks.

4.4. Determination of ploidy level

To determine the level of ploidy of affected *L. asarifolia* plants, was used flow cytometry method.

Leaf sample was taken from each cultured plant. The leaf of *L. asarifolia*, approximately 1 cm², was placed in small petri dish with 1 ml of Otto I solution (Tween 20 + citric acid) and chopped into smallest particles. The resulting solutions were filtered through a nylon filter (50 µm) into a small test tube. To the filtrate tube was added 1 mL of Otto II + DAPI (Na₂HPO₄ · 12 H₂O + 4', 6-diamidino-2-phenylindole [fluorescent dye]).

The resulting solution was measured in a flow cytometer.

5. Results

By polyploidisation were affected 180 plants in total. During the experiment plants began to branch and the resulting number of affected plants was 324. Every leaf sample were analysed and compared to control sample. The success of the polyploidisation was tested by flow cytometry. The best result has shown on plants affected by 40 μM concentration for 48 hours (Tab 1).

In total were obtained 9 mixoploid plants: most of all (3 plant) in concentration 40 μM for 48 hours, 2 plants in 40 μM for 24 hours, 2 plants 80 μM for 24 hours and 2 in 80 μM for 48 hours. None of the tetraploid plant were obtained.

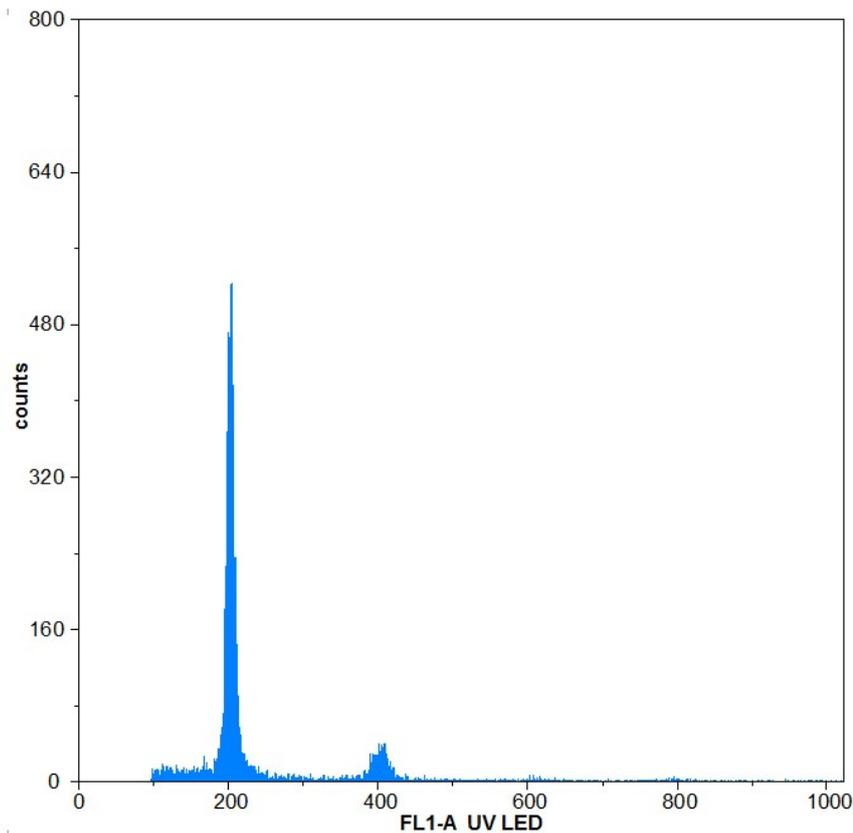


Fig 7. Histogram of control (diploid) plant, peak on channel 200 correspond to the nuclei of the control plant

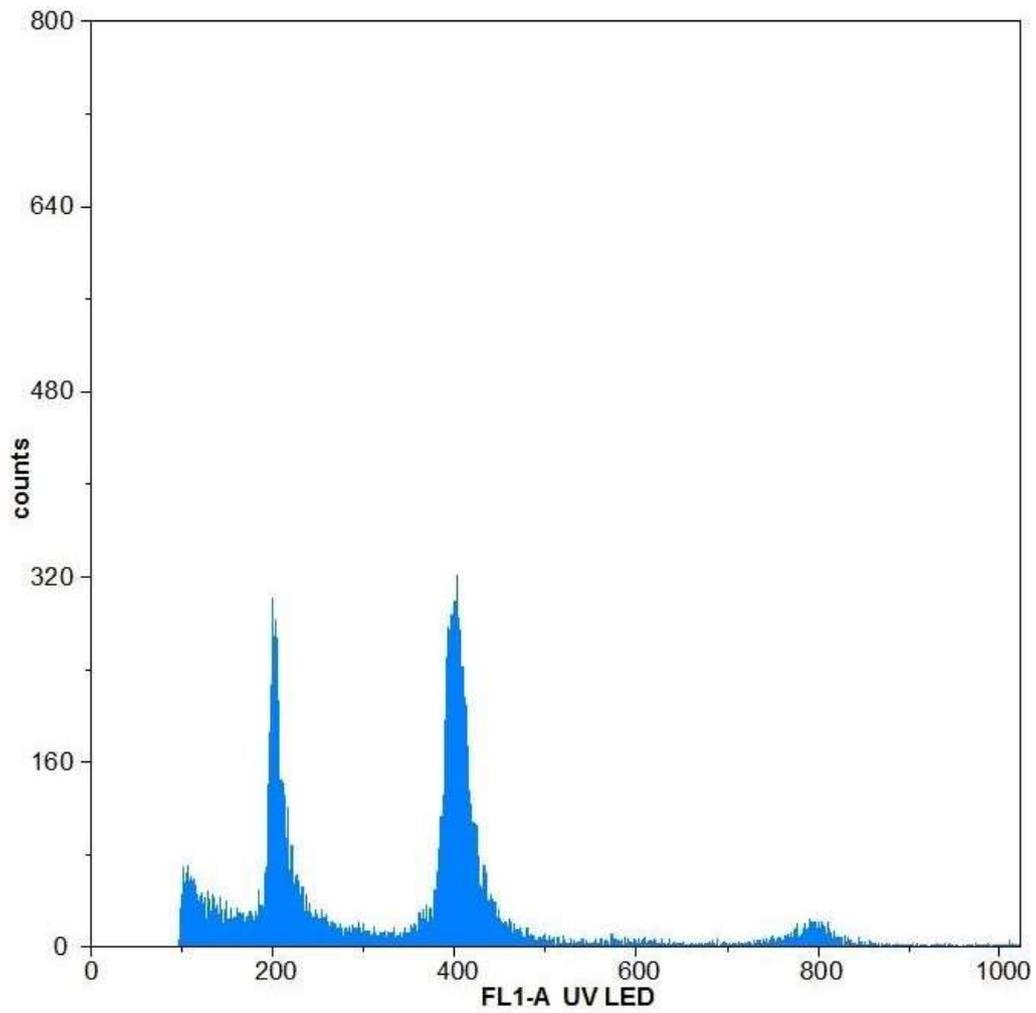


Fig 8. Histogram of the mixoploid plant affected by 40 concentration for 24 hours.

Author: author's own elaboration (2020)

Table 1: Influence of oryzalin effect on survival of shoots rate *in vitro*, and number of polyploids of *L. asarifolia*

Concentration of oryzalin	Duration of influence	Number of nodal segments	Number of plants at the end of experiment	Survive rate	Number of tetraploid plants	Number of mixoploid plants	Polyploid ization efficiency
(μM)	(Hours)			(%)			(%)
20	24	30	74	100	0	0	0
	48	30	55	100	0	0	0
40	24	30	54	100	0	3	5,5
	48	30	64	100	0	2	3,125
80	24	30	34	100	0	2	5,88
	48	30	43	100	0	2	4,65
In total		180	324	100	0	9	

Author: author's own elaboration (2020)

5.1. Morphology evaluation

New obtained polyploid plants were cultivated on 25 ml of MS medium without any additional grow regulators. The control diploid sample was also observed. Every plant was placed in individual Erlenmeyer flask and cultivated for 3 weeks. While growing process morphology characteristic were evaluate and compared with control plant.

The highest plant was plant treated in concentration 40 μM for 24 hours, 2 cm higher in comparison with control plant (7 cm), all the rest 8 plants were approximately the same size

During the branching mixoploid plants produce up to 5 stolons (plant treated in 80 μM for 48 hours), then the control plant maximum 2. The number of nodes on stems did not differ among all the samples and control plants. As usual it was one or two nodes per stem.

The greatest number of leaves was count on plant treated in 80 μ M for 48 hours, 7 grown leaves and 5 new young leaves grown from buds

The biggest diameter of leaf was observed on plant treated in 40 μ M for 48 hours (3 cm x 3,5 cm), in comparison to the control plant (2 cm x 2 cm). Other plants had their leaves sizes close to control plant. Also, at most mixoploid plant was observed a leaf dysmorphia. One side of mixoploid plant is noticeably longer (Fig 11), or typical cordate shape (Fig 9) of plant was almost absent (Fig 10). Some leaves tips have changed from rounded tip to acute (Fig 12).

The roosts of mixoploid plants were more branched and diversified then control plant roots, although all plant had approximately same length.

Although tetraploid plants were not obtained by oryzalin affection, mixoploid plants were obtained, so hypothesis H0 is confirmed, oryzalin is an effective anti-mitotic agent for induction of polyploidy *in vitro*. And confirmed the hypothesis H1, because morphological difference was observed between control (diploid) plants and mixoploid plants. Developed mix ploidy plants from experiment could be used for further studies of morphological and phytochemical properties



Fig 9. Leaf of control plant



Fig 10. Plant treated in 40 μM for 24 hours



Fig 11. Plant treated in 80 μM for 24 hours



Fig 12. Plant treated in 40 μM for 48 hours

Author: author's own elaboration (2020)

6. Discussion

Induced polyploidisation in *Lycianthes asarifolia* by oryzalin needs to be studied further. For the family *Solanaceae* Juss., this method was used to induce polyploidisation in seedlings of tomatillo (*Physalis ixocarpa* Brot.) (Robledo-Torres et al. 2011).

Robledo-Torres et al. (2011) performed an attempt to induce polyploidy in seedlings of *Physalis ixocarpa* Brot. ($2n = 2x = 24$). The aim of studies was to increase its breeding potential by developing autotetraploids through colchicine-based induction. Seedlings of the Rendidora cultivar were treated with colchicine in concentrations of 0.04%, 0.08%, 0.12%, 0.16% and 0.20% for 24 hours. For each treatment 50 seeds were germinated in Petri dishes with filter paper soaked with distilled water for at a temperature range of 23-26°C for 48 hours. When the seeds started to break their coats, colchicine was applied. After 24 h, the seeds were sown in 200-well polystyrene trays with peat moss in 1:1 proportion. Then developed platelets gained approximately height of 12 cm. they were translated to and experimental field.

The greatest success was found after 0.12% and 0.16% of colchicine treatment, 67% and 65%, respectively. The 50% rated success was observed for concentrations of 0.08% and 0.20%.

Considering the results of the first set of concentrations, the experiment was repeated with the best colchicine concentrations, being 0.12% and 0.16%. As polyploids candidates for meiotic analysis were chosen plants at flowering stage with rounder, darker and bigger leaves than the control type.

Tetraploid plants have shown the phenotype difference from diploid control plant. Also, polyploid plants were bigger in size and had longer life cycle (Robledo-Torres et al. 2011).

In result of present bachelor thesis, mixoploid plants of *Lycianthes asarifolia* are higher in size and have larger leaves with dysmorphic changes. Although due to time conditions evaluated plants were not at full growth size.

In this bachelor thesis was performed an attempt to induce polyploidisation in the species *in vitro* induction polyploidy of *Lycianthes asarifolia* (Kunth & Bouché) Bitter

using three concentrations of oryzalin 20 μM , 40 μM , 80 μM acting for two time periods 24 and 48 hours.

The results demonstrate that action of 40 μM of oryzalin for 48h induced polyploidisation in three plants (with 5,5% rate of success), two plants were found in 40 μM for 24 hours (3,125% rate of success), two plants 80 μM for 24 hours and 2 in 80 μM for 48 hours (5,88 % and 4,65 rate of success, respectively). All examined plants were considered as mixoploids and none of the examined plant were tetraploid

In Wang et al. (2011) research seeds of *Lycium barbarum* (*Solanaceae*) were treated with a mixture of colchicine and DMSO for different times. In solution of 0.05% colchicine and 2% DMSO for 48 hours the way was developed mixoploids with 53.3% rate of success. Also new obtained mixoploid plant demonstrated better morphologic properties (thicker and stronger stems, larger leaf area, thriving vegetative growth) (Wang et al. 2011).

Also studies of *In Vitro* Polyploidisation in *Solanum lycopersicum* Mill. with colchicine, showing mixoploid occurrence in variety of different concentration (1.5 mM/96 h or 1.5 mM/120 h, 2.5 mM/96 h, 5.0 mM/96 h, 6.5 mM/120 h, and 8.0 mM/96 h) (Cola et al. 2014). However, morphology of different polyploids was not evaluated and compared in this research.

In already mentioned studies of *Physalis ixocarpa* Brot. polyploidisation, with the great morphology changes, fruit yield, between tetraploid and diploid plants was not significantly different (Robledo-Torres et al. 2011).

Due to ploidy instability, many studies disregard mixoploid plantlets. However, mixoploids can be used as explant source as well, and may contribute to tissue culture research (Cola et al. 2014).

In present bachelor thesis of polyploidisation of *L. asarifolia*, were obtained only mixoploid plant with new morphological properties in comparison with control diploid plants. For further studies of polyploidy induction of *L. asarifolia*, studies of oryzalin action in different concentration and time durations are essential.

7. Conclusion

Using induced polyploidisation *in vitro*, were obtained mixoploid plants of *Lycianthes asarifolia* (Kunth & Bouché) Bitter with new morphological properties. This method was first time used in this species. Although, oryzalin is seems to be an effective anti-mitotic agent for the induction of polyploidy in *L. asarifolia*, further studies of its action are required. Because this species is widely used in Latin America as a fruit plant, less as medicinal and ornamental herb, the newly acquired genotypes required biochemistry and nutritional studied *in vitro* as well and should be monitored and evaluated *in vivo*. The obtained results extend the variability of *Lycianthes asarifolia* (Kunth & Bouché) Bitter.

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Appendices

List of the Appendices:

- I. Composition of MS culture medium (Murashige - Skoog 1962)

Appendix I: Composition of MS culture medium (Murashige - Skoog 1962)

Médium Murashige – Skoog			
	Nutrient solutions up to 1 l dest. H ₂ O	Weights per 1 l of nutrient solution	Measure the per 1 l (pH 5.7)
A	NH ₄ NO ₃ KNO ₃ CaCl ₂ MgSO ₄ · 7 H ₂ O KH ₂ PO ₄	16,5 g 19 g 3,3 g 3,7 g 1,7 g	100 ml
B	H ₃ BO ₃ MnSO ₄ · 4 H ₂ O (H ₂ O) ZnSO ₄ · 4H ₂ O (7H ₂ O)	620 mg 2,23 g (1,69 g) 860 mg (1, 06 g)	10 ml
C	KI NaMoO ₄ · 4 H ₂ O	83 mg 23 mg	10 ml
D	CuSO ₄ · 5 H ₂ O CoCl ₂ · 6 H ₂ O	2,5 mg 2,5 mg	10 ml
E	NA ₂ EDTA FeSO ₄ · 7 H ₂ O	3,72 g 2,78 g	10 ml
V	Nicotinic acid Pyridoxin (B6) Thiamin (B1) Glycin (aminoacid)	50 mg 50 mg 10 mg 200 mg	10 ml