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Micropropagation and polyploidization of *Tacca integrifolia*

Master's thesis

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Declaration

I hereby declare by my signature that this master's thesis „Micropropagation and polyploidization of *Tacca integrifolia"* is my original work, where I am using cited references, which are written in the end of the thesis.

In Prague 20.4. 2018

…………………………

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Abstract

Tacca integrifolia is a tropical ornamental plant with possibility of using in gardens and interiors as well. The objectives of this thesis were to develop a suitable protocol for micropropagation and mitotic polyploidization protocol using two antimitotic agents oryzalin and trifluralin. To carry out the multiplication experiment, shoots were cultivated on MS (Murashige & Skoog, 1962) culture medium supplemented with cytokinins BAP and zeatin at concentrations 0.3-2.0 mg. l^{-1} alone or in combination with NAA at concentration 0.1 mg. l^{-1}. Optimal treatment proved to be 1.0 mg. l^{-1} BAP producing the highest number of adventitious shoots (10.38±1.19 shoots per explant). Rooting of shoots was tested on MS medium supplemented by auxin NAA at concentrations 0.1 and 0.3 mg. l^{-1}. Higher number of roots was obtained from treatment 0.1 mg. l^{-1} NAA (3.18±0.45 roots per explant). A total of 29 well-rooted plants were transferred *ex vitro*, to the greenhouse conditions; a survival rate of 86.2% was obtained after eight weeks of evaluation. Genetic stability was verified through ISSR markers and flow cytometry. Three ISSR primers showed no polymorphism among plants tested. Similarly, no changes in ploidy level during the micropropagation process was detected via flow cytometry. To induce polyploidy, 160 plants were treated with oryzalin and trifluralin at concentration 25 and 35 µM in 24 and 48 h of exposure time. Ploidy level of all regenerated explants was measured using flow cytometry. The highest efficiency in ploidy induction was achieved in treatment 25 µM oryzalin applied for 48 h, where 20% of tetraploid plants were obtained and survival rate reached 95%. Total production of artificial induced tetraploids was 13.75%. Tetraploid plants displayed lower growth and leaf morphological changes. In this study, micropropagation protocol was optimized. This technique can serve as an efficient tool for commercial production of genetically identical plant. Morhoplogical changes in tetraploids can be beneficial in breeding of *Tacca integrifolia*. However, thorough morphological study of tetraploid plants is further needed.

Keywords: flow cytometry, micropropagation, molecular markers, polyploidy, *Tacca integrifolia*

Abstrakt

Tacca integrifolia je tropická okrasná rostlina s využitím v zahradách i interiérech. Cílem této práce byla optimalizace protokolu pro *in vitro* mikropropagaci a vytvoření metodiky pro navození mitotické polyploidie v *in vitro* podmínkách za pomoci dvou antimitotických činidel oryzalinu a trifluralinu. Nejprve bylo kultivační Murashige a Skoog (1962; MS) médium obohaceno o růstové regulátory BAP a zeatin v koncentracích 0,3-2,0 mg. l^{-1}. Dále byly využity tyto koncentrace cytokininů (BAP a zeatin) v kombinaci s auxinem NAA o koncentraci 0,1 mg. l^{-1}. Varianta média s přídavkem 1,0 mg. l^{-1} BAP byla vyhodnocena jako nejlepší z hlediska nejvyššího počtu nově vytvořených odnoží (10.38±1.19 nové odnože na explantát). V následující fázi byly rostliny pěstovány na médiu s obsahem auxinu NAA pro podporu zakořeňování, a to v koncentracích 0,1 a 0,3 mg. l^{-1}. Více kořenů na rostlinu (3.18±0.45 kořene na rostlinu) bylo získáno na médiu s 0,1 mg. l^{-1} NAA. Celkově 29 zakořeněných rostlin bylo převedeno *ex vitro* do skleníku. O dva měsíce později procento přežití rostlin dosahovalo 86,2 %. Genetická stabilita jedinců získaných v rámci optimalizované mikropropagace byla ověřena pomocí tří ISSR primerů, které nedetekovaly žádný polymorfismus napříč testovanými rostlinami. Stabilní úroveň ploidie byla potvrzena pomocí průtokové cytometrie. V rámci pokusného navození *in vitro* polyploidie u *T. integrifolia* byly rostliny byly vystaveny působení antimitotických činidel oryzalinu a trifluralinu o koncentracích 25 a 35 µM po dobu působení 24 a 48 hodin. Detekce polyploidie byla realizována pomocí průtokové cytometrie. Výsledky průtokové cytometrie ukázaly, že nejvyšší úspěšnost polyploidizace byla u varianty 25 µM/48 h oryzalinu, kde bylo získáno 20 % tetraploidů a procento přežití rostlin po aplikaci oryzalinu dosahovalo 95 %. Prvotní hodnocení tetraploidní rostliny ukázalo, že tetraploid je nižšího vzrůstu a jeho listy se velikostně i morfologicky liší od diploidních rostlin. Mikropropagaci optimalizovanou v rámci této studie lze doporučit jako efektivní způsob množení druhu *T. integrifolia*, kdy je možno v krátkém čase získat velké množství geneticky uniformních rostlin. Naproti tomu morfologické změny u polyploidních rostlin mohou být využity ve šlechtění tohoto druhu. Podrobnější hodnocení polyploidních genotypů bude předmětem dalšího výzkumu.

Klíčová slova: průtoková cytometrie, mikropropagace, molekulární marker, polyploidizace, *Tacca integrifolia*

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List of abbreviations

AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
BAP	6-benzylaminopurine
bp	base pairs
CULS	Czech University of Life Sciences Prague
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleotide acid
FISH	florescent *in situ* hybridization
FTA	Faculty of Tropical AgriSciences
GISH	genome *in situ* hybridization
ISSR	inter simple sequence repeat
MS	Murashige and Skoog medium (1962)
NAA	α-naphthaleneacetic acid
PCR	polymerase chain reaction
PGR	plant growth regulator
RAPD	random amplification of polymorphic DNA
RFLP	restriction fragment length polymorphism
SNP	single nucleotide polymorphism
SSR	simple sequence repeat

1. Introduction and literature review

Tacca integrifolia is an ornamental and medicinal plant from family Dioscoreaceae with pantropical distribution through Southeast Asian regions (Dahlgren et al. 1985). It is perennial, rhizomatous, and short-stemmed herb with large showy inflorescences (Drenth 1972). It is used as ornamental plant in botanical gardens and households as well as an outdoor plant (Plantrescue 2018). *T. integrifolia* also has medicinal potential, especially in traditional medicine as treatments for e.g. gastric ulcer, controlling blood pressure and enteritis (Chuakul et al. 2000).

The species is naturally propagated by seeds and adventitious shoots, however, propagating by seeds is complicated due to long term germination and low germination index (Plantrescue 2018; Noor Anilizawatima et al. 2013). These methods of propagation have a low efficiency. Hence, micropropagation is suitable way to overcome these problems. Micropropagation enable to obtain a substantially larger amount of uniform, virus-free plants in short time (Loberant & Altman 2010). Despite its advantages, micropropagation process may have few hindrances, among them somaclonal variation (Phillips et al. 1994). Flow cytometry and polymerase chain reaction (PCR)-based techniques as ISSR and random amplification of polymorphic DNA (RAPD) markers are the most suitable for assessment of *in vitro* regenerants, because they require only a small amount of deoxyribonucleotide acid (DNA), they are simpler and faster than DNA-based markers as amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) (Reddy et al. 2002).

Current genetic variability within *T. integrifolia* species is quite low; only two different forms, one from Malaysia and one from Tibet, were discovered (Zhang et al. 2011). Genetic variability can be extended via breeding techniques e.g. selection or polyploidization. Polyploids have doubled number of chromosomes, which can lead to modified sizes of morphological organs, intensification of flower colour and earlier flowering (Takamura & Miyajima 1996; Zahumenicka et al. 2017). Inducing polyploidy could improve ornamental characteristics of *T. integrifolia* and polyploidization of plants could be an efficient tool to obtain new cultivars.

The aims of this thesis were to develop an appropriate protocol for micropropagation of *T. integrifolia* and to induce *in vitro* mitotic polyploidization in this species.

1.1 Taxonomy and relative species of *Tacca integrifolia*

Dioscoreales is order comprising family Dioscoreaceae, where *T. integrifolia* belongs. The order Dioscoreales is divided into three families: Dioscoreaceae, Burmanniaceae, Nartheciaceae. According to recent information, the family Dioscoreaceae comprises four genera: *Dioscorea*, *Stenomeris*, *Tacca* and *Trichopus* (including genus *Avetra*). This family is typical for having simultaneous microsporogenesis, thickened seed endotesta, climbing habit (lost in genus *Tacca*) and *Dioscorea*-type leaf petioles (Caddick et al. 2002).

In some previous studies, genera *Avetra*, *Stenomeris* and *Trichopus* belonged to their own families (Hutchinson 1959; Takhtajan 1987; Huber 1991). *Tacca* genus had its own family Taccaceae. However, it was placed to Dioscoreaceae (Dumortier 1829). *Tacca* differs from the other Dioscoreaceae by its stemless habit, however it has a lot of uniform characteristic as tuberous underground parts rich in steroidal saponins, or petiolate and reticulate-veined leaves. *Tacca integrifolia* and *Tacca chantrieri*, which have entire-leaves, are resembled to the *Trichopus zeylanicus* (Caddick et al. 2002). The showy pseudo umbellate inflorescence can interconnect *Tacca* with Amaryllidaceae (Dahlgren et al. 1985).

According to the Caddick et al. (2002) *Tacca* genus comprises ten well-known species: *T. bibracteata*, *T. celebica*, *T. chantrieri*, *T. ebeltajae*, *T. integrifolia*, *T. leontopetaloides*, *T. palmata*, *T. plantaginea*, *T. palmatifida* and *T. parkeri*. These species could be classified into three groups according to the type of their inflorescences: A. petty inflorescences with small bracts and short bracteoles e.g. *T. leontopetaloides*, *T. plantaginea* and *T. parkeri*, B. inconspicuous inflorescences with bracts but without bracteoles e.g. *T. palmata* and *T. palmatifida* and C. very significant inflorescences with large bracts and long bracteoles which are observed in plants as *T. integrifolia* and *T. chantrieri* (Zhang et al. 2011).

1.2 Origin and geographical distribution of *Tacca* spp.

The family Dioscoreaceae where *Tacca integrifolia* belongs comes from Southeast Asia, which is considered as the centre of biodiversity of this plant (Dahlgren et al. 1985). The species are distributed through wet and seasonally dry tropics and few exceptions of species are distributed also in temperate and alpine regions (Caddick et al. 2002).

The place of *Tacca* species origin is unknown. However, the area of distribution covers area between tropics of Cancer and Capricorn (Drenth 1976). *Tacca* genus has pantropical distribution with the centre in Indo-Malaysia – from Southeast Asia to the Solomons islands. Nine species come from this place. *Tacca* genus is divided in two groups according to their distribution. First group, 4 entire-leaved species, which comprises also *T. integrifolia* (Figure 1), is distributed from East India to West Java. The second group, 4 palmate-leaved species, is distributed in the area from Indo-China to the Caroline Island. There are exceptions as *T. leontopetaloides*, distributed through Africa from west coast to Easter Island and *T. parkeri*, which is endemic in tropical South America (Drenth 1972).

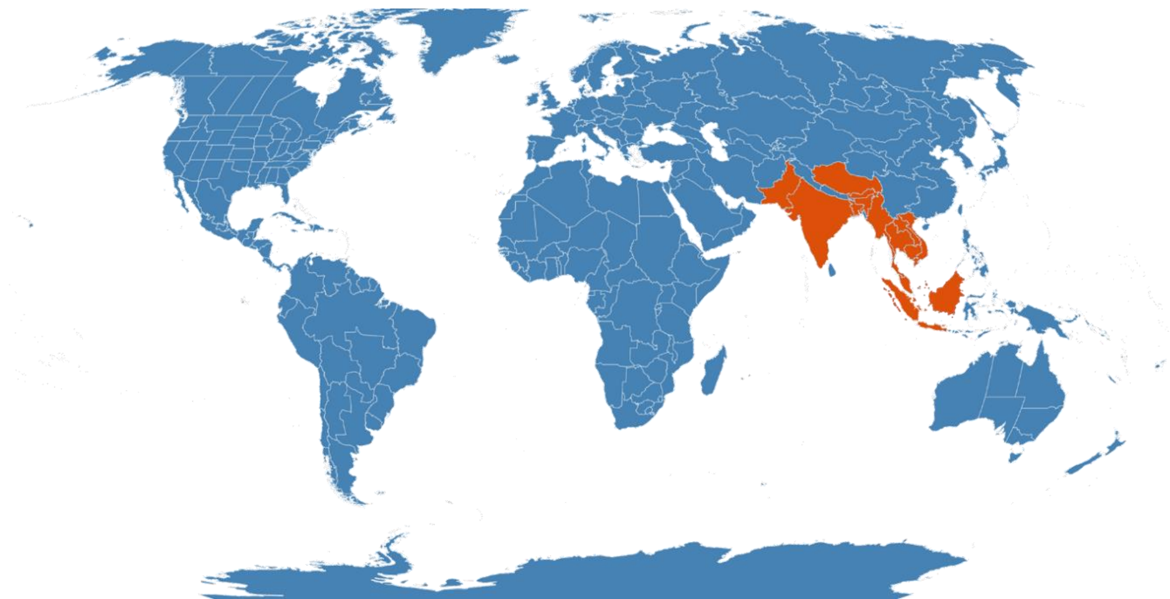

Figure 1. Geographical distribution of *Tacca integrifolia* (Leonard 2007)

1.3 *Tacca integrifolia*

1.3.1 Ecology

Tacca integrifolia is a perennial herb occurring in humid tropical and subtropical lowland evergreen primary or secondary forests, especially on steep slopes, ridges or near water. There is also possibility to find the plants rarely in open vegetation, e.g. on roadsides and clearings, and coastal areas (Drenth 1972; Endress 1995). Its occurrence moves from sea level up to 1,200 m, rarely to 1,500 m altitude. Plants from the hills are usually larger than those from the plains (Drenth 1972).

The plant grows on various types of soils, e.g. sandy or stony substrate, limestone, or red earth, but usually in place with high amount of decayed organic material (Drenth 1972; Plantrescue 2018). Shaded and warm area is usually places where it can be found, it thrives under 70-80% shade (Meerow 1995). It also thrives in temperate areas during warm and humid conditions from spring to autumn. During tropical winter or dry season, leaves usually start to dry and yellowing. If the plant has proper conditions, it does not need a resting period (Plantrescue 2018).

1.3.2 Botanical and morphological description

1.3.2.1 Habit

Tacca integrifolia is a herbaceous lily-like plant (Figure 2; Saw 1993). It is long-lived, stemless, rhizomatous plant (Drenth 1972). It grows vertically, and its height ranges from 60 cm to 120 cm. Plant has narrow to medium hypogeal stems. *T. integrifolia* is bigger and showier than more common *Tacca chantrieri* (Missouri Botanical Garden 2018; Plant Rescue 2018).

Figure 2. Habit of *Tacca integrifolia* (Logees 2018)

1.3.2.2 Foliage

Besides its showy inflorescences, *Tacca* also has attractive evergreen foliage (Figure 3; Missouri botanical garden 2018; Plantrescue 2018). *T. integrifolia* has entire leaves, very variable, usually ovate, or lanceolate (Drenth 1972). Leaves are petiolate, reticulate-veined and they are organized in basal rosette (Dahlgren et al. 1985), growing from the top of the rhizome (Plantrescue 2018). Leaves can grow up to 60 cm in length and 25 cim wide with petiole length up to 40 cm. *Tacca* usually has a small number of leaves (Drenth 1972; Missouri botanical garden 2018; Plantrescue 2018).

Figure 3. Leaves of *Tacca integrifolia* (Author)

1.3.2.3 Inflorescence and fruit

Tacca integrifolia has the largest flowers from the whole genus (Logees 2018). (Figure 4; Missouri botanical garden 2018). *T. integrifolia* has actinomorphic and hermaphroditic flowers (Zhang et al. 2005).

Inflorescences of *T. integrifolia* contain two bracts growing up to 22 cm, which are white with purple venation and are situated above the purple flowers and long hanging white whisker-like filiform bracteoles. Each flower can grow up to 3.2 cm wide and is arranged in cluster (Figure 3; Flora & Fauna 2018; Meerow 1995; Plantrescue, 2018; Zhang et al. 2011). *Tacca* starts to bloom, when produces 2-3 full sized leaves and it usually blooms from the May to November (Meerow 1995). Each plant can produce from 6 up to 12 flower stems during the warm months.

Fruits are capsules about 4 cm long usually ranging green to black with fleshy pericarp. It needs up to one year to ripen (Figure 4; Chee et al. 2013; Drenth 1972; Plantrescue 2018).

Figure 4. Inflorescence of *Tacca integrifolia*; A., D. Whole inflorescences with bracts and bracteoles, B. single purple flower, C. whole inflorescences backside, E. fruit (Flickr 2018; University of Connecticut 2018; Flora & Fauna 2018)

1.3.2.4 Rhizomes

The tuberous rhizomes of *T. integrifolia* are naked, caliginous, and starchy. There are three types of rhizomes in *Tacca* genus: I. A vertical elongate rhizome with apical growth II. A roundish rhizome with an apical cavity representing the growth

centre III. A horizontal elongate rhizome from the upper part. *T. integrifolia* has the first type of rhizomes, i.e. cylindric and thick rhizome with apical leaves, which grows vertically up to 12 cm long (Figure 5; Efloras 2018; Drenth 1972).

Figure 5. Rhizome of *T. integrifolia* (Big plant nursery 2018)

1.3.3 Reproductive biology

The mating system of *Tacca integrifolia* is lack of information however, study from Xiong et al. (2006) focused on population genetic structure of *T. integrifolia* in Tibet and Malaysia indicates that *T. integrifolia* is primarily a self-fertilized plant. However, it was reported that high selfing of *T. integrifolia* is associated with infrequent cross-pollination (Cruden 1977; Lloyd 1980; Barrett 2001).

It was observed that dark floral colours, the presence of long bracts, no nectar secretion, lack of fragrance and a decaying odour are well-known features of the sapromyiophilous syndrome, which can be connected with fly pollination and it can be found in other families of flowering plants, e.g., Asclepiadaceae, Aristolochiaceae, Araceae, Orchidaceae (Endress 1995; Fægri & van der Pijl 1971; Proctor et al. 1996). The plant is attractive for insects of the order *Diptera*, only under these circumstances. *T. integrifolia* produces compounds attracting flies but not perceptible by human (Proctor et al. 1996). According to the Lim and Ragusot (2017), the most common visitors of *T. integrifolia* are individuals of the insect family Ceratopogonidae. Some of

them left the plant with trapped pollen. Nevertheless, the relationships between floral morphology and pollinators requires a more detailed exploration (Zhang et al. 2011).

1.3.4 Uses and properties

Tacca integrifolia is an ornamental plant with specific unusual flowers and showy foliage (Missouri botanical garden 2018). This plant is suitable as a houseplant and for outdoor conditions, but not as cut-flower, because it has quick tendency to wilting (Plantrescue 2018). It has become one of the favourite potted-plant as well as outdoor plant in Malaysia; where it has high potential to increase profits in floriculture industry (Abdullah et al. 2013). *Tacca* has also become increasingly popular through the world trade with ornamental flowers (Zhang et al. 2007).

T. integrifolia has been also used in traditional medicine in tropical regions of Asia (Su 1997). It has been used as treatment for gastric ulcer, enteritis, hepatitis, controlling blood pressure, improving sexual function, heart failure and kidney diseases (Chuakul et al. 2000; Jamaludin & Jamaludin 2016). According to the Malaysian traditional medicine, paste from tubers is used to rash by insect bites (Tropical spice garden 2016). *Tacca* is also used against hair fall in Borneo (Drenth 1972).

1.3.5 Diversity and plant breeding of *Tacca integrifolia*

Information about origin, morphological, ecological and uses characteristic of *T. integrifolia* are known (Drenth 1972). Despite of increasing demand of *T. integrifolia* in floriculture industry, there is lack of information about plant diversity (Abdullah et al. 2013). There is only one study focused on diversity of this species, which compare *T. integrifolia* from Malaysia and from Tibet (Zhang et al. (2011). Large differences in morphological characters e.g. different colour of bracts and flowers indicated that they may be accepted as two separate species (Zhang et al. 2011).

Potential breeding possibilities comprise different type of selection, hybridization and polyploidization (Stebbins 1956; Encyclopedia Britannica 2018). From several studies focused on polyploidization in ornamental plants it is well known, that this technique provides efficient tool to improve ornamental and thus commercial potential of these plants, e.g. *Cyclamen* (Takamura & Miyajima 1996), *Hibiscus acetosella* (Contreras et al. 2009) Polyploidization led to increased size of flowers,

intensification of inflorescence colour, earlier flowering, higher compactness of plant and other morphological differences compared to diploid plant (Takamura & Miyajima 1996; Contreras et al. 2009; Zahumenicka et al. 2017) Polyploidization was also successfully used in breeding of tuber crops from Dioscoreaceae family, i.e. *Dioscorea rotundata* and *Disocorea cayenensis* where polyploidization increase size of leaves as well as size of stomata which serve as source of genetic variability in breeding programmes (Babil et al. 2016).

1.3.6 Plant husbandry

1.3.6.1 Cultivation

Tacca may be cultivated as an outdoor as well as indoor plant. It is shade-loving plant, so it prefers to grow in low light or 70-80% shade; however, it can also tolerate heavy shade (Logees 2018; Meerow 1995; Missouri botanical garden 2018; Plantrescue 2018). It requires temperature between 21-32 °C and high air circulation (Plantrescue 2018). *Tacca* is native to tropical rain forests, hence it has high requirements for consistent moisture (Missouri botanical garden 2018). Higher humidity than 50% is optimal for this plant; it can be replaced with humidifiers. More intensive watering is necessary during growing period (warm months). Rainwater is more suitable than tap water for watering, because tap water contains chlorine and fluoride, which can lead to browning of leaves. However, soil in pots should dry out during the cold months.

Tacca thrives on rich, well-drained soil enriched of compost or leaf litter. In case of pot cultivation, soil mixture can be made by 60% peat moss, 30% perlite and 10% vermiculite or also substrate for orchids is also suitable (Logees 2018).

T. integrifolia is relatively easy to grow plant; cultivation of this plant is easier than of more common *Tacca chantrieri* (Missourri botanical garden 2018; Plantrescue, 2018).

1.3.6.2 Fertilization

T. integrifolia is sensitive to strong fertilizers, hence it prefers controlled-release fertilizer. Salts fertilizers can dehydrate plant, which can lead to browning of leaves and burning of roots. However, it is tolerant to the different commercial plant fertilizers and organic fertilizers. *T. integrifolia* needs biweekly fertilising during the growing season

with a diluted liquid or orchid fertilizers. Fish-based fertilizers are also suitable, especially for promoting flowers (Plantrescue 2018).

1.3.6.3 Pests and diseases

T. integrifolia is almost pest and disease-free plant. There are only some exceptions as snails slugs, spider mites and caterpillars, which are eating its leaves; however damage is usually minimal (Meerow, 1995). Concerning the diseases, only gray mold could be more serious for plant (Missouri botanical garden 2018).

1.3.6.4 Propagation

White bat flower can be propagated from seed or stem budding. Propagation by stem budding can be done during repotting. Each adventitious shoot might be transferred into individual pots. Propagation by seeds is more complicated, because of low germination rate of seeds (Charoensub et al. 2008). Firstly, seeds must be cleaned from the fleshy pericarp, dried, and quickly sowed. They are sowing into germination medium and covered. It is necessary to keep them in warm and moist conditions without direct sun. Germination takes 8-12 weeks, then seedlings are transferred to the individual pots, until each seedling has at least first well-developed leaf. This process is time consuming. Micropropagation represents more efficient method to obtain high amount of geneticly uniform plants in short time (Charoensub et al. 2008; Engelmann 2011; Plantrescue 2018).

1.4 Micropropagation

Micropropagation is suitable technique to obtain a large amount of virus-free plants. Micropropagation brings approach for large-scale plant multiplication of true-to-type plant or superior varieties in horticulture industry focusing on ornamental plants. (International Atomic Energy Agency 2004; Engelmann 2011). The whole process of cultivating cells or organs of selected plant is carried out in aseptic condition. It is one of *in vitro* technologies, which combines effect of explant source, type of plant and treatments (Debergh & Read 1991; Loberant & Altman 2010).

Micropropagation is divided into five stages (Figure 6), and three of them are carried out *in vitro* (Debergh et al. 1990; Loberant 1994; Pierik 1987). According to

Debergh and Maene (1981), first stage 0 is used for selection and preparation of explant source. The aim of stage 0 is ensuring of high hygienic conditions for mother plant, which can reduce possibilities of future contamination in stage I. (Debergh et al. 1990; Read 2007). The purpose of stage I. is to establish viable explant or seed in culture (Altman & Loberant 2010). Stage II. is about rapid regeneration of new regenerants. Stage III. Is intended for establishment of well-rooted plantlets prepared for *ex vitro* transfer (Altman & Loberant 1998). Last stage IV. is *ex vitro* transfer and acclimatization of plantlets in *ex vitro* conditions (Dubuc & Desjardins 2007; Pospisilova 2007). Each stage has its own specific requirements (Loberant & Altman 2010).

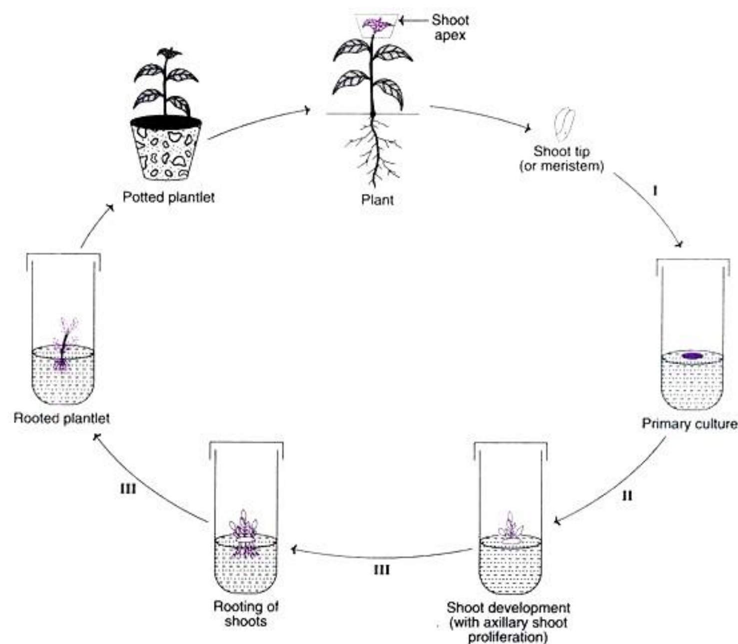

Figure 6. Stages of micropropagation (Jha 2017*)*

On the other hand, this process can be complicated by contamination of plant, which causes economic losses (Cassells 1988), or potential occurrence of somaclonal variation (Sasson 1993). According to Dubuc and Desjardins (2007) transfer of plant material from stage III. to stage IV. can also cause high losses because of acclimatization of plant to the different conditions.

1.4.1 Micropropagation in Dioscoreaceae family and in *Tacca* genus

Plants from family Dioscoreaceae, where *Tacca* belongs, are important staple crops for their nutritional content in tubers. Unfortunately, *in vivo* production of plants in Disocoreaceae is complicated by pathogens, which attack plants. Additionally, infection can persist in subsequent generation and reduce the quality of plant material. Conventional propagation by seeds is slow and inadequate for high yield (Saleil et al. 1990; Ng 1992; Mitchell & Ahmed 1999; Das et al. 2013).

Micropropagation is a tool to overcome these problems (Vaillant et al. 2005). This technology was already applied in some *Dioscorea* species as *D. abyssinica* (Martine & Cappadocia 1991), *D. alata* (Mantell & Hugo 1989), *D. batatas* (Koda & Kikuta 1991), *D. weightuu* (Mahesh et al. 2010), *D. floribunda* (Sengupta et al. 1984).

In case of *Tacca* genus, studies on micropropagation for two species i.e., *Tacca leontopetaloides* (Borokini et al. 2011; Cepkova et al. 2015; Martin et al. 2012) and *Tacca chantrieri* (Charoensub et al. 2008) are available. Each study mentions problems with slow and inefficient germination of seeds in this genus. Each species can be also propagated by shoots or tubers (Spenemann 1994; Borokini et al. 2011; Charoensub 2008), but this technique cannot produce a massive amount of plants (Engelmann 2011). Nevertheless, micropropagation in *Tacca* is the most suitable method to overcome problems with low production (Noor Anilizawatima et al. 2013).

In case of *T. integrifolia*, Noor Anilizawatima et al. (2013) developed a protocol for better seeds germination. Where the seeds germinated *in vitro* after 21 days instead of 1-9 months with using conventional methods. Mentioned study also recommended 6-benzylaminopurine (BAP) for seed germination.

1.5 Somaclonal variation and assessment of genetic fidelity of *in vitro* regenerants

In vitro micropropagation offers possibility of high production of true-to-type plants from the selected mother plants in a short time (Lee 1988). However, utilization of this technique for commercial production may be complicated by somaclonal variations, which has been reported in several crops (Phillips et al. 1994).

Somaclonal variation is exhibited as cytological abnormalities, phenotypic mutation, sequence change, and gene activation or gene silencing. Epigenetic changes

can be activated through the culture process or may also be involved in cytogenetic instability through modification of heterochromatin, and as a basis of phenotypic variation through the modulation of gene function (Kaeppler et al. 2000).

Somaclonal variability includes also changes in chromosome number (Mujib et al. 2007; Leva et al. 2012). Differences are occurring during the first few mitotic divisions of callus formation. During growth of callus or liquid cell cultures composing of chromosomes could be changed (O'Connor & Fitter 1983). Type of morphogenesis (direct/indirect), type of tissue, explant source, media components and duration of cultivating cycle could by factors inducing variation during *in vitro* culture (Pierik 1987).

The occurrence of somaclonal variability can limit chance of sustainability of micropropagation system (Salvi et al. 2001).

1.6 Molecular markers and assessment of genetic fidelity

Markers can be divided into morphological and molecular, where morphological markers are detected at the first sight, but molecular markers are expressed at the molecular level i.e. DNA or proteins (Singh et al. 2008). Then, molecular markers can be divided into three main groups: I. Hybridization-based markers as restriction fragment polymorphism (RFLP), II. PCR-based markers: random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellite or simple sequence repeat (SSR) and last III. Sequence-based markers: single nucleotide polymorphism (SNP; Varshney et al. 2007).

It suitable tool for detect genetic variations among close related species such as two isogenic lines (Reddy et al. 2002; Singh et al. 2008). RFLP is not suitable for high number of samples and RAPD could not be transferable between laboratories, while SSRs and AFLPs are efficient in identifying polymorphism and SSR is more easily automated (Shariflou et al. 2001).

1.6.1 Assessment of genetic fidelity using ISSR markers

Among the DNA-based markers, AFLP and RFLP are able to prove genetic fidelity, however they are often inappropriate due to use of radioactive labelling, expensive enzymes and extensive labour. On the other hand, PCR-based techniques as

ISSR and RAPD require only a small amount of DNA sample, do not require radioactive probes and are simple and fast (Reddy et al. 2002). The ISSR is based on dinucleotide, tetranucleotide or pentanucleotide repeats (Zietkiewicz et al. 1994). The ISSR markers have been already used to detect the somaclonal variations in ornamental species such as *Ceropegia panchganiensis* (Chavan et al. 2013) or *Zingiber rubens* (Mohanty et al. 2011).

1.7 Polyploidy

Polyploid organisms contain multiple sets of chromosomes compared to the diploids, which contain two complete sets of chromosomes (Kohmetscher et al. 2012). In natural way, polyploids can arise by chromosomes duplication of somatic cell or during meiosis due to failure of one or more pairs of homologous chromosomes (Ramsey & Schemske 2002).

Almost half of angiosperms are polyploids (Grant 1963). Natural polyploidy can elongate life cycle and higher competitive ability (Lumaret and Guillerm 1997). There are several differences in morphology, ecology (resistance to environmental stresses), physiology and cytology between diploid and polyploid plants (Dewitte et al. 2009; Kaensaksiri et al. 2011; Kohmetscher et al. 2012). Some other advantages over diploidy is increasing of allelic diversity and heterozygosity which can be used for breeding purposes and genetic studies, otherwise polyploids of some species could be self-incompatible (Udall & Wendel 2006). In addition, genomic multiplication apparently augments the production of secondary metabolites (Zahed et al. 2010; Zahedi et al. 2014).

Examples of common natural polyploids include wheat, sisal, coffee, banana, potatoes, and maize, cotton, oats, and sugar cane (Leitch & Leitch 2008).

1.7.1 Mitotic polyploidization and role of antimitotic agent

There is possibility to induce artificially polyploidy in plants using antimitotic agents (Ramsey & Schemske 1998). Blakeslee and Avery (1937) started with artificial mitotic polyploidization technique and effectively applied in agriculture in the 1930s. However, the first published practice of *in vitro* polyploidization was in tobacco

(Murashige & Nakano, 1966). Polyploids can be created by mitotic polyploidization, which is based on the doubling of somatic tissues (Ramsey & Schemske 1998).

The synthetic production of chromosome doubled *in vitro* plants can be achieved by manipulation of the plant's cell cycle. The plant cell cycle is divided in different phases: a G1-phase (post-mitotic interphase), an S-phase (DNA synthesis phase), a G2-phase (pre-mitotic interphase) and a M-phase (mitosis, Francis 2007). The cell cycle can be disrupted by a variety of chemicals which are called antimitotic agents. Only those, which affect the cell cycle at the end of the S-phase and before the start of cytokinesis (during the late stages of the M-phase) are suitable for chemically induced polyploidization (Dewitte & Murray 2003).

M-phase (Figure 7) is process of duplication and distribution of chromosomes and it is divided into four stages as prophase, metaphase, anaphase, and telophase. Metaphase is point of interest for polyploidization, because antimitotic agents (known also as metaphase inhibitors) can inhibit the formation of a spindle fibre that results to inseparable chromosomes and increasing of ploidy level. Antimitotic agent causes the dissolution (depolymerization) of protein microtubules, which make up the mitotic spindle in dividing cells. This leaves the cell with doubled sets of chromosomes (Encyclopedia Britannica 2017; Planchais et al. 2000).

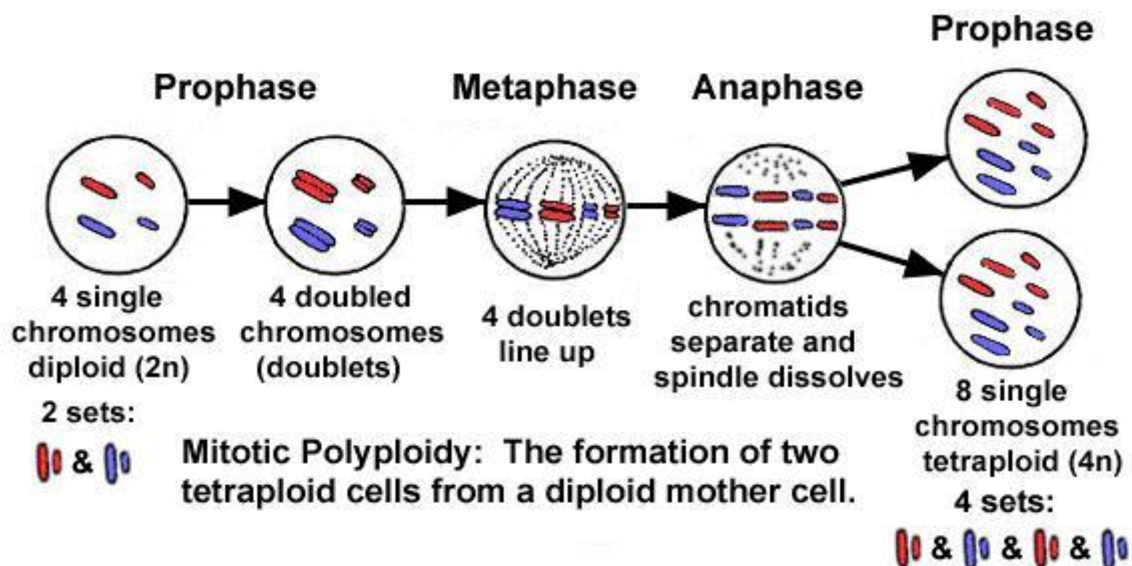

Figure 7. Phases of mitotic polyploidization (Armstrong 2002)

Antimitotic agents interrupt the metaphase by connection the a- and b-tubulin dimers, which decrease the possibility attachment of new dimers on the assembly side of the microtubule, without reducing degradation of the microtubule in the end of the disassembly (Devine et al. 1993; Vaughn 2000). In this case disassembly is faster than assembly and microtubules are depolymerized. The common antimitotic agents are colchicine and dinitroanilines which comprise oryzalin and trifluralin (Devine et al. 1993).

The most commonly used antimitotic agent is colchicine - toxic alkaloid extracted from the seeds and bulbs of the *Colchicum autumnale* (Eigsti & Dustin 1955; Nilanthi et al. 2009). An advantage of colchicine is that it does not reduce its polyploidizing capacities after sterilizing in autoclave (Xiong et al. 2007). Nevertheless, it is necessary to use high concentration of colchicine, due to worse binding to plant tubulins. Additionally, even if the polyploidization is successful, there is high risk of side effects often leading to plant sterility, abnormal growth, and gene mutations. High toxicity to humans, due to high affinity to microtubules of animal cells, was reported. These all above mentioned reasons are leading to finding an alternative cell cycle inhibitor (Dhooge et al. 2011; Luckett 1989; Morejohn et al. 1984).

Initial strategy is to check if a compound, which is toxic for plants as a herbicide, may act as an antimitotic agent. It was detected that 25% of all common herbicides affect process of mitosis. Herbicides have two main advantages as better binding on plant tubulin, and lower toxicity to humans compared to the colchicine (Bajer & Molebajer 1986; Hugdahl & Morejohn 1993; Morejohn et al. 1987). Due to above reasons, some herbicides were considered as alternatives to colchicine (Bartels & Hilton 1973; Hess & Bayer 1974). Oryzalin and trifluralin have become the most common alternatives to colchicine (Dhooghe et al. 2011).

1.7.2 Methods of polyploidy detection

Methods of polyploidy detection can be divided into nuclear DNA estimation molecular analyses and cytological estimation (Gupta 2007).

Karyological analysis and observation of chromosomal changes using light microscopy and oil immersion has been successfully used for detecting somaclonal

variation *in vitro* regenerants (Al-Zahim et al. 1999; Raimondi et al. 2001; Mujib et al. 2007), however, this technology is time-consuming (Doležel 1997).

The most widely method used in plants is flow cytometry (Bennett & Leitch 2005). Flow cytometry has several steps including isolation of nuclei from leaf tissue by chopping, staining of isolated nuclei with florescent dye, which binds to DNA and then passed through a flow cytometry. Then nuclei go through the lights, lenses, mirrors, and amplifiers, which detect and convert fluorescent light, which is emitted by each nucleus as digital signal. The whole DNA content can be detected by comparing of fluorescence intensity and plant known DNA amount (Dar & Rehman 2017).

There is also possibility to use molecular methods for detecting of polyploidy. Firstly, molecular cytogenetic techniques as florescent in situ hybridization (FISH) and genome in situ hybridization (GISH), are common for analysing of genome organization in different species (Dar & Rehman 2017). The molecular mapping can be used for analysing of ploidy level, however it is complicated in comparison to diploids, because large population need segregation analyses. Thus, the statistical analysis can be complicated to obtaining correct genetic distance (Porceddu et al. 2002).

1.7.3 Polyplodization in ornamental plants

Artificially induced polyploidization is suitable tool to produce new plant materials for breeding purposes. New induced polyploids are not a completely new genetic material, but it is an organism with additional copies of existing chromosomes and genes, which can result in phenotypic variation (Adams & Wendel 2005; Wang 2017).

Besides increasing of genetic variability, polyploidy can be useful in floriculture mainly to increase the size of plant parts, especially flower and leaves (Wimber & Wimber 1967; Zahumenicka et al. 2017). Other changes such as deeper-coloured flowers, intensification of colour, fragrance and earlier flowering can be observed (Dermen 1940; Takamura & Miyajima 1996; Zahumenicka et al. 2017).

However, polyploidization may have also disadvantages as slower growth or problems with roots development (Griesbach 1981).

Artificial polyploidization is well-known tool especially in orchid industry and has an important influence to produce superior cultivars (Kamemoto 1961).

Polyploidization of cyclamens led to enlargement of flowers and intensification of their colour, as well as to physiological changes associated with better ability to accumulate chalcone (Takamura & Miyajima 1996). In *Hibiscus and Anemone* genus, polyploid plants had increased size of their organs, but *Hibiscus* decreased its habit (Contreras et al. 2009; Zahumenicka et al. 2017).

2. Aims of the thesis

This thesis is focused on two main objectives. First objective is to assess the effect of plant growth regulators on inducing of new shoots and roots, and to develop suitable protocol for micropropagation of ornamental plant *Tacca integrifolia*. The second objective is the induction of polyploidy in *T. integrifolia* to increase ornamental value of this species.

The objectives can be divided into six following parts:

- Optimization of direct shoot organogenesis
- Optimization of *in vitro* rooting
- *Ex vitro* transfer of well-rooted plants
- Detection of the genetic fidelity of *in vitro* plantlets using molecular markers and flow cytometry
- Induction of *in vitro* polyploidy
- Detection of ploidy level using flow cytometry

Optimization of all phases of micropropagation and polyploidization would increase production and commercial potential of this ornamental species.

The main aims were determined based on three hypotheses:

H1: Plant growth regulators support development of new shoots and new roots of different plant species and thus they will induce shoots and roots development in *T. integrifolia.*

H2: *In vitro* direct morphogenesis enables to obtain genetic uniform plants; therefore, genetic fidelity will be verified in *T. integrifolia.*

H3: Mitotic polyplodization will be induced by using antimitotic agents in *Tacca integrifolia*. Oryzalin and trifluralin induced mitotic polyploidization in wide spectrum of plant species.

3. Material and Methods

3.1 Plant material

To carry out the experiment, aseptic culture of *Tacca integrifolia,* established within previous experiments in the Laboratory of Plant Tissues Cultures at the Czech University of Life Sciences, Faculty of Tropical AgriSciences (FTA), was used. Initially, seeds obtained from Universitatea Bades–Bolyai Gradina Botanica „Alexandru Borza "in Romania in 2013, via index seminum, were used.

3.2 Methodology

3.2.1 Initial *in vitro* multiplication of plants for the experiment

To obtain sufficient amount of *in vitro* plants for the experiment, *in vitro* multiplication of plants was carried out in Laboratory of Plant Tissue Cultures, Department of Crop Sciences and Agroforestry, Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague. Each plant approximately 0.5 cm in height with main shoot and without any adventitious shoots, leaves and roots was cultivated on Murashige and Skoog medium (MS; 1962) medium supplemented with 100 mg. l^{-1} *myo*-inositol, 30 g. l^{-1} sucrose, 8 g. l^{-1} agar, with addition of 0.5 mg. l^{-1} of BAP for quick development of new shoots. Plants were sub-cultured every four weeks. Plants were cultivated at 25/23 °C under 16 hours of light and 8 hours dark regime with 36 µmol m^{-2}. s^{-1} fluorescent light.

3.2.2 Micropropagation of *Tacca integrifolia*

3.2.2.1 *In vitro* propagation of *Tacca integrifolia*

For precise experiment, plants of the same size, 0.5 cm in height, without leaves, roots and with no adventitious shoots were used (Figure 8). For each treatment, MS medium supplemented with 30 g. l^{-1} sucrose, 100 mg of *myo*-inositol and 8 g. l^{-1} of agar and different type and concentrations of plant growth regulators (PGRs) was used. pH of medium was adjusted to 5.7 and medium was sterilised in autoclave at temperature 121 °C in 1.1 Pa for 20 minutes. In this experiment, two different cytokinins at various concentrations were tested. Zeatin and BAP at concentrations 0.3, 0.7, 1.0, 1.5 and 2 mg. l^{-1} individually or in combination with 0.1 mg. l^{-1} α-naphthaleneacetic acid (NAA)

were added to the medium. Twenty plants were used per each treatment in two repetitions. As control treatment, MS medium without any PGRs, was used. Plants were cultivated in 100 ml Erlenmayer flasks at 25/23 °C under 16 hours of light and 8 hours dark regime with 36 µmol m^{-2}. s^{-1} fluorescent light. Shoot formation, plant height, number of leaves and roots formation were evaluated after eight weeks of cultivation. Special characteristics (growth of callus at the basis of explants, colour changes of leaves and vitrification) were observed as well.

Table 1. Treatments for multiplication of *Tacca integrifolia* plants

BAP (mg. l^{-1})	Zeatin (mg. l^{-1})	NAA (mg. l^{-1})
0.3		
0.7		
1.0		
1.5		
2.0		
	0.3	
	0.7	
	1.0	
	1.5	
	2.0	
0.3		0.1
0.7		0.1
1.0		0.1
1.5		0.1
2.0		0.1
	0.3	0.1
	0.7	0.1
	1.0	0.1
	1.5	0.1
	2.0	0.1
0	0	0

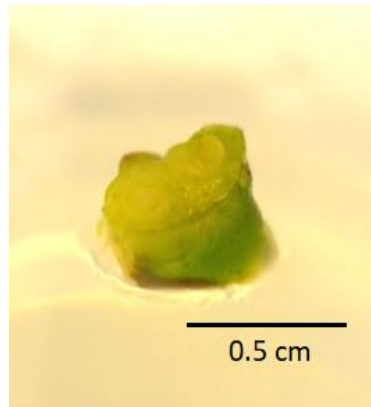

Figure 8. An initial explant
used for *in vitro* propagation (Author)

3.2.2.2 *In vitro* rooting and *ex vitro* transfer

For *in vitro* rooting, shoots (0.5 cm in height) without leaves, and adventitious shoots were used. Auxin NAA at concentrations 0.1 mg. l^{-1} and 0.3 mg. l^{-1} was used to induce rooting. Medium supplemented with 30 g. l^{-1} sucrose, 100 mg of *myo*-inositol and 8 g. l^{-1} of agar and without any PGRs, was used as control. Plants were cultivated in 100ml Erlenmayer flasks for eight weeks in 16/8 h light or dark regime at 25/23 °C at a photosyntetic photon flux density of 35 µmol m^{-2}. s^{-1} provided by fluorescent tubes. Each treatment consisted of 20 plants in two repetitions. The number and length of roots were evaluated after eight weeks of *in vitro* cultivation.

Well-rooted plants (at least three roots more than 1.5 cm in length) were chosen for *ex vitro* transfer. The *ex vitro* transfer was carried out at the Botanical garden of the FTA in Czech University of Life Sciences Prague (CULS) in 2018.

At the beginning, plants were taken out of the Erlenmeyer flasks and roots were carefully washed from residues of culture medium under tap water. After thorough washing, plants were transferred to pots with sterile mix of perlite-garden substrate in ratio 1:1. Pots with plants were then transferred to tightly close small greenhouse to maintain high air humidity. Greenhouse was opened after a couple of days. Survival rates were evaluated after eight weeks.

3.2.2.3 Statistic evaluation

Statistical analysis evaluated all results obtained from the process of micropropagation. Statistical analysis of data consisted of analysis of variance (ANOVA) and the significantly different means were identified using the Tukey´s HSD test ($p = 0.05$) [StatSoft STATISTICA 12.0].

3.3 Evaluation of *in vitro* regenerants

3.3.1 DNA isolation

DNA isolation and ISSR analysis were carried out in Laboratory of Molecular Biology of CULS, 2018.

For ISSR analysis, 18 randomly chosen plants regenerated on MS medium supplemented by 1.0 mg. l^{-1} BAP were used. The plant from control treatment was used as control treatment. 100 mg of fresh leaves per one plant were used to lyophilisation. Lyophilized leaves were used for DNA isolation. For isolation genomic DNA from lyophilized and crushed leaves, CTAB (Cetyltrimethylammonium Bromide) method was used (Doyle and Doyle 1989) with addition trace of polyvinylpyrolidon (PVP) and 5 µl of RNase of concentration 10 mg. μl^{-1} (Thermo Scientific, Czech Republic). Resulting concentration of DNA was measured on UVS-99/UVISDrop (Avans Biotech, Taiwan) and confirmed by electrophoresis on 1% agarose gel. The isolated DNA was diluted to 80 ηg. μl^{-1} and stored at -20 °C in freezer.

3.3.2 ISSR analysis

For ISSR screening, set of 3 primers, which showed clear and scorable bands was used (Zietkiewicz et al. 1994). PCR amplification was mixed in total volume 20 µl containing 10 µl of PPP Mastermix [150 mM Tris-HCl, pH 8.8 (25 °C), 40 mM $(NH_4)^2SO_4$, 0.02% Tween 20, 5 mM $MgCl_2$, 400 µM dATP, 400 µM dCTP, 400 µM dGTP, 400 µM dTTP, 100 U. ml^{-1} Taq-Purple DNA polymerase, monoclonal antibody anti-Taq (38 nM)], stabilisers and additives (Tob-Bio, Czech Republic), 0.2 µl of Bovine serum albumin (Thermo Scientific, USA 0.5 µl of selected primer and 7.3 of doubled distilled H_2O. In this phase, samples were put on ice and 2 µl of isolated DNA of *T. integrifolia* were added in the amplification solution. PCR process was executed in Veriti 96 Well Thermal cycler (Applied Biosystems, USA). For annealing temperature

optimization wasa range between 47-54 °C was used. The cycling conditions were divided into several steps: initial denaturation for 5 minutes at 95 °C, 40 cycles of denaturation at 94 °C for 1 minute, following by annealing in range of temperature 47-54 °C for 1 minute and extension 72 °C for 2 minute including final extension at 72 °C for 8 minutes. PCR amplified products were separated on 1% agarose gel in 1 × TBE buffer using 40 minutes, 100 V and 120 mA on electrophoresis. Ethidium bromide (Carl Roth GmbH, Germany) stained the agarose gel, which was then visualized under UV light in the Syngene GENi2 UV transilluminator (Trigon plus, Czech Republic). The size of products was measured using 100 base pairs (bp) ladder GeneRuler 100 bp Plus DNA Ladder (Thermoscientific, Lithuania). Results of ISSR analysis was evaluated as presence (1) or absence (0) bands in the gel. Only, well visible bands were evaluated.

3.3.3 Measurement of relative DNA content

Detection of DNA ploidy levels was carried out in Laboratory of Plant Tissue Cultures, FTA in Prague 2017. For detection of ploidy level, 40 plants cultivated on medium with addition 1.0 mg. l^{-1} as well as, and one control plant, were used. *Glycine max* 2C = 2.50 pg (Dolezel et al. 1994) was used as internal reference standard. For analysis of ploidy level, the modified two-step methodology according Doležel et al. 2007 was used. Firstly, approximately 1 cm^2 of leaves *T. integrifolia* was chopped by razor blade on Petri dish together with leaves of internal reference standard *Glycine max*. Tissue was mixed with 1 ml of cold Otto buffer I solution containing 0.1 M citric acid and 0.5% Tween 20 (Otto 1990). Then suspension containing isolated nuclei was filtered through a 42 µm nylon mesh into test cuvette and pure solution was mixed with 1 ml of staining solution containing DAPI stock (4,6-Diamidino-2-phenylindole) and Otto buffer II. containing 0.4 M $Na_2HPO_4.12H_2O$ (Doležel & Göhde, 1995). In all samples, relative fluorescence intensity of at least 3,000 nuclei was measured using CyFlow Space flow cytometer. The results were processed by the FlowMax software (Partec GmbH, Münster, Germany).

The DNA-ratios were evaluated in each analysed sample by dividing the mean of the G0/G1 peak of the *Tacca integrifolia* sample by the mean of the G0/G1 peak of the internal standard.

3.4 Polyploidization of *Tacca integrifolia*

Antimitotic agents oryzalin and trifluralin at different concentrations and time duration were used for polyploidy induction in the culture of nodal segments. Oryzalin and trifluralin were used as water solutions with 2% dimethyl sulfoxide (DMSO) to help antimicrotubular agents penetrate through cell walls. Solutions were mixed in aseptic conditions in flow box and DMSO had the sterilization effect on the solution. Two concentrations, 25 µM and 35 µM in two different time of duration 24 h and 48 h, were tested (Table 2). Each treatment consisted of 20 plants.

Table 2. Concentrations and exposure time of antimitotic agents used for *in vitro* polyploidization of *Tacca integrifolia*

Oryzalin (µM)	Trifluralin (µM)	Treatment time (h)
25		
35		
	25	
	35	24 h
25		
35		
	25	
	35	48 h

Initially, plants per one treatment were deprived of leaves, roots and adventitious shoots, alternatively, and were sub-cultured on the MS medium, supplemented with 100 mg of *myo*-inositol, 30 g. l^{-1} sucrose, and 8 g. l^{-1} of agar, in the 1l volume beaker for two days.

Next, the explants were overlaid by solutions of oryzalin (Figure 9) or trifluralin for 24 h or 48 h and were cultivated under the same conditions as described above. After 24/48h, plants were washed three times from residue of antimitotic agent in sterile distilled water. Regenerating shoots from axillary buds were cut off and explants were sub-cultured on the fresh MS medium to the 100 ml Erlenmeyer flask. This part of

experiment was carried out in Laboratory of Plant Tissue Cultures of FTA in CULS and Crop Research Institute during year 2017.

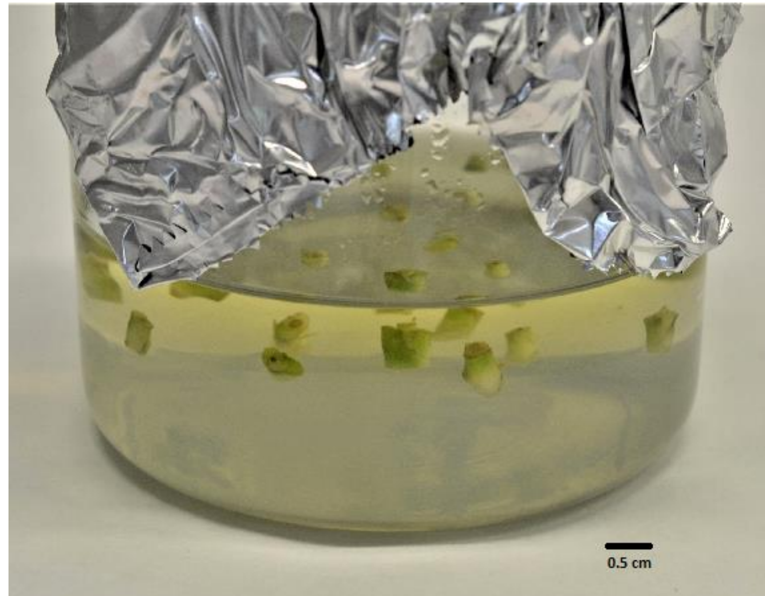

Figure 9. Shoots *of Tacca integrifolia* on MS medium overlaid by oryzalin solution (author)

3.4.1 Analysis of ploidy level using flow cytometry

Detection of DNA ploidy levels was carried out in Laboratory of Plant Tissue Cultures, FTA in Prague 2017. For detection of ploidy level, 132 plants treated with microtubule agents, and one control plant, were used. The entire methodology is described in chapter 3.3.3. Measurement of relative DNA content.

4. Results

4.1 Micropropagation

4.1.1 *In vitro* propagation of *Tacca integrifolia*

Plants were cultivated on MS medium supplemented by two different cytokinins – BAP and zeatin at concentrations 0.3-2.0 mg. l^{-1} either alone or in combination with NAA at concentration 0.1 mg. l^{-1} (Table 3). The most efficient treatment for *in vitro* propagation of *T. integrifolia* was 1.0 mg. l^{-1} of BAP (Table 3; Figure 11) where plants produced substantially higher number (10.38 shoots per explant) of adventitious shoots compared to other treatments.

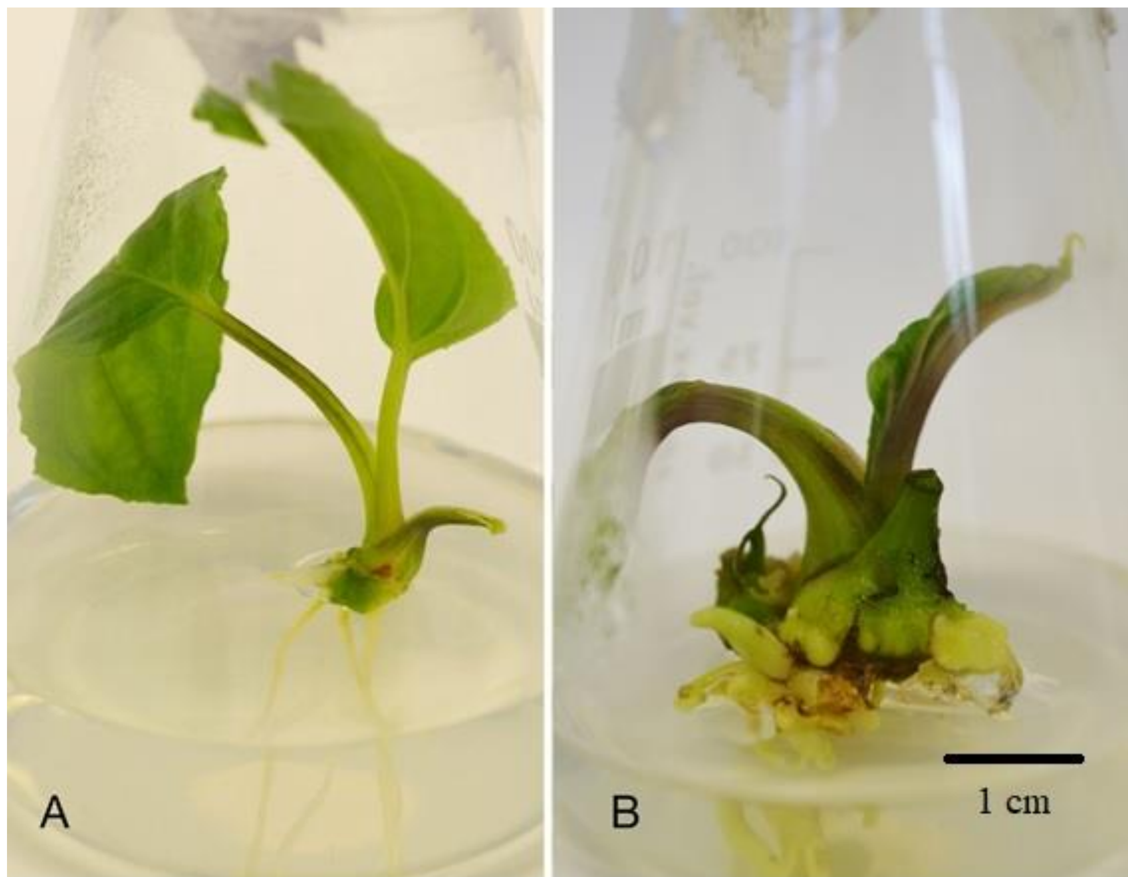

Figure 10. Comparison of explants from two different medium A. An explant on control MS medium without PGRs; B. An explant on MS medium supplemented by 1.0 mg. l^{-1} BAP

It was evaluated that the intermediate concentration of BAP was the most suitable for *in vitro* propagation. Lower BAP concentrations decreased number of

produced adventitious shoots. Therefore. plants on MS medium (control treatments) produced only 1.95 new shoots per explant. On the contrary, higher concentrations of BAP led to formation of week plants, morphological abnormalities, and callus was produced on the basis of the explant (Figure 11).

Figure 11. Callus formation on medium supplemented with 1.5 mg. l^{-1} BAP

Apparently with increasing concentration of BAP the height of plant was decreased. Thus, the highest average height of plants on BAP (80.1 mm) was achieved on medium with the lowest concentration of BAP (0.3 mg. l^{-1}). This average height was comparable to the average height of plants from medium supplemented with 1.0 mg. l^{-1} zeatin.

Plants on media with zeatin were higher, of healthy appearance, but they did not produce high number of adventitious shoots. Among concentration of zeatin, the best results in terms of number of shoots were obtained on medium with 1.5 mg. l^{-1} zeatin, where 4.50 shoots per explant were regenerated. The lowest efficiency in shoot production at lowest concentration of zeatin was observed. Altough, plants did not

produce callus in medium with addition of zeatin but leaves from plants started to brown in few cases.

The addition of auxin NAA to the optimal BAP concentration did not increase the number of adventitious shoots. The combination of cytokinin and auxin had positive effect on regeneration of new adventitious shoots, especially in case of BAP, where the best treatment 1.5 BAP mg. l^{-1} + 0.1 NAA mg. l^{-1} allowed production of 8.05 new shoots per explant, while the lowest yield was observed in combination 0.3 mg. l^{-1} BAP + 0.1 mg. l^{-1} NAA. The highest concentration (2.0 BAP mg. l^{-1} + 0.1 NAA mg. l^{-1}) again lead to the callus production. The highest average height was 75.0 mm in concentration 0.7 mg. l^{-1} zeatin and the lowest was 33.2 mm in the highest concentration i.e 2.0 mg. l^{-1} of BAP. In case of zeatin the highest result was 4.52 new adventitious shoots per explant from treatment 0.7 mg. l^{-1} of zeatin, which is more than in the case of zeatin without NAA and from lower concentration. However, it is possible to see a downward trend in case of plant height.

Overall, the whole production of new shoots and plant height was lower than in the case of cytokinins without NAA. Plants were lower, unhealthy, and started to produce callus. Thus, the latter combination was the least effective from the whole micropropagation process. In almost all cases, plants achieved better results in case of cytokinins without NAA, there is no need to combine cytokinins and auxins in case of *T. integrifolia*.

Some peculiarities as differentiation or producing of new shoots from petioles was observed in older cultures on medium with addition of cytokinin, regardless of concentration used (Figure 12).

Table 3. Results of influence PGRs on production of new adventitious shoots

	Treatment concentrations (mg. l^{-1})	**No. of shoots mean±SE**
BAP	0.3	7.88±0.91 ab
	0.7	4.75±0.87 bcde
	1.0	10.38±1.19 a
	1.5	7.10±1.22 abc
	2.0	6.70±1.21 abcd
Zeatin	0.3	1.70±0.42 e
	0.7	2.30±0.67 de
	1.0	2.77±0.75 cde
	1.5	4.50±1.26 bcde
	2.0	3.06±0.73 cde
BAP + 0.1 NAA	0.3 + 0.1	1.03±0.27 e
	0.7 + 0.1	1.92±0.61 e
	1.0 + 0.1	4.73±1.36 bcde
	1.5 + 0.1	8.05±1.40 ab
	2.0 + 0.1	5.07±1.45 bcde
Zeatin + 0.1 NAA	0.3 + 0.1	1.19±0.37 e
	0.7 + 0.1	4.52±0.90 bcde
	1.0 + 0.1	2.50±0.50 cde
	1.5 + 0.1	1.97±0.55 de
	2.0 + 0.1	2.27±0.58 de
Control	0	1.95±0.77 e

* Mean values in a column, followed by different letters, were significantly different according to the Tukey's HSD test ($P \leq 0.05$).

** SE standard error

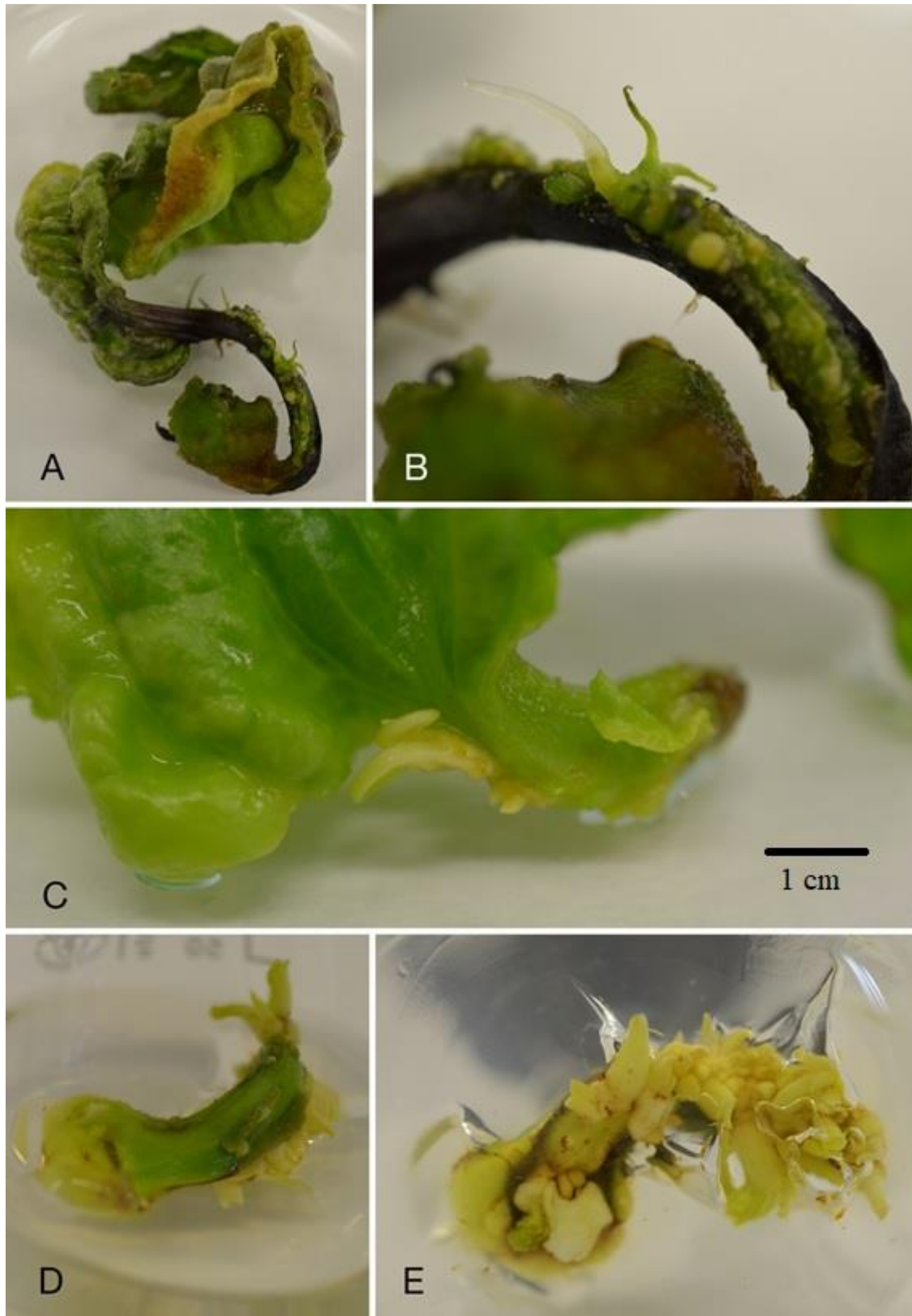

Figure 12. Plants producing shoots from petioles. A., B. *in vitro* regenerants on medium supplemented by 1.0 mg. l^{-1} zeatin; C., D., E. *in vitro* regenerants on medium supplemented by 1.0 mg. l^{-1} BAP (Author)

4.1.2 *In vitro* rooting and *ex vitro* transfer

Almost no rooting was observed on the most appropriate media for *in vitro* propagation. Hence, auxin NAA was used as suitable PGR to support *in vitro* rooting. Lower concentration of NAA used (0.1 mg. l^{-1}) was more efficient in terms of number of roots (3.18 roots per explant) than higher concentration (0.60 roots per explant) and control treatment. However, the length of roots was superior from control treatment compared to the treatment with 0.1 mg. l^{-1} NAA. The average length of roots was 15.4 mm (Table 4). In lower concentration of NAA, 76.9% of plants produced roots, while in higher concentration, the efficiency was only 12.5%. On the other hand, 57.5% plants on control medium regenerated roots. Some plants started to produce callus and some plants had adventitious shoot.

Table 4. Results of influence NAA on roots induction

	Treatment concentration (mg. l^{-1})	**Number of roots mean±SE**	**Length of roots (cm) mean±SE**	**Rooting efficiency (%)**
NAA	0.1	3.18±0.45 a	1.51±0.10 a	76.9
	0.3	0.60±0.34 b	0.23±0.11 b	12.5
Control	0	2.54±0.43 a	1.54±0.12 a	57.5

* Mean values in a column, followed by different letters, were significantly different according to the Tukey's HSD test ($P \leq 0.05$).

** SE standard error

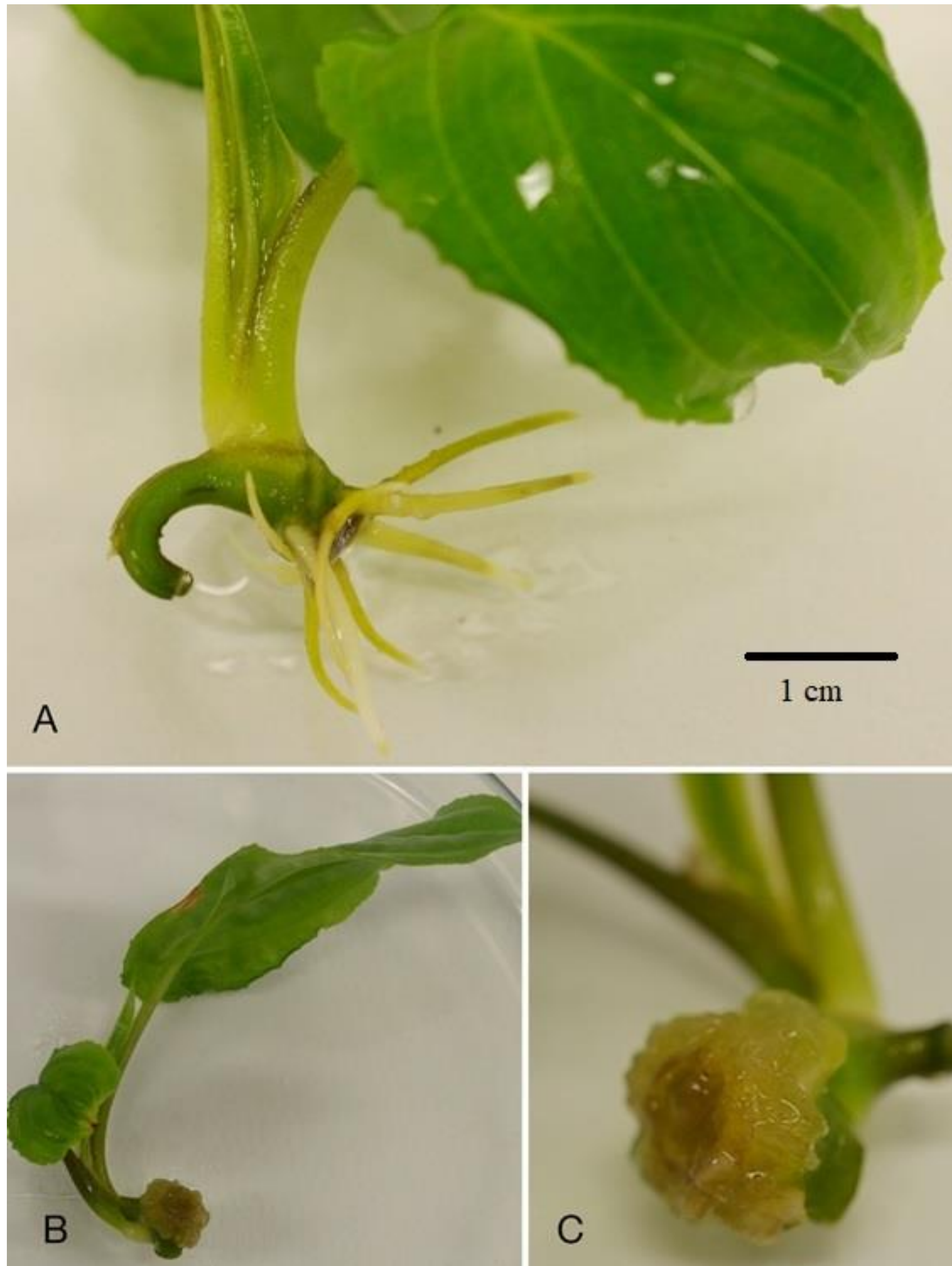

Figure 13. Plants from medium supplemented with NAA. A. Well-rooted plant from medium with addition 0.1 mg. l^{-1} NAA. B., C. Plant from medium with addition 0.3 mg. l^{-1} started to produce callus instead of roots (Author)

In total, 29 well-rooted and healthy plants (17 from 0.1 mg. l^{-1} NAA and 12 from control treatments) were transferred *ex vitro*, to the Botanical garden, FTA, CULS.

After eight weeks, plants regenerated new leaves and whole growth was restored. No morphological abnormalities were observed. A total of 25 plants survived and acclimated to the new conditions (Figure 14).

Figure 14. Plants after *ex vitro* transfer. A., B. Plants transfered to the pots. B. One week after *ex vitro* cultivation. C. Plantlet of *Tacca integrifolia* two months after *ex vitro* transfer (Author)

4.1.3 Assessment of genetic stability of *in vitro* regenerants

Within primary study of assessment of genetic fidelity, the DNA was successfully extracted from 19 samples of *in vitro* regenerants where 18 samples regenerated from medium supplemented with 1.0 mg. l^{-1} BAP and one sample was control plant. Extracted DNA was used for confirmation of genetic fidelity of

regenerated shoots using the ISSR primers. The number of evaluated and well visible bands ranged from two to four per one primer. In total 9 amplification fragments ranging from 450 to 1,500 base pairs (bp), were obtained from all samples (Table 5). The amplified products of all samples were monomorphic. The used ISSR primers did not reveal any somaclonal variation (Figure 15).

Table 5. ISSR primers, annealing temperatures, numbers, and sizes of amplified fragments

Primer code	Annealing temperature (°C)	Total No. of bands amplified	No. of evaluated bands per primer	Rate of polymorphic bands per primer (%)	Range of amplification (bp)
UBC 809	54	57	3	0.00	500-1,500
UBC 812	49	86	4	0.00	450-900
UBC 824	53	38	2	0.00	300-700
Total	-	181	9	-	-

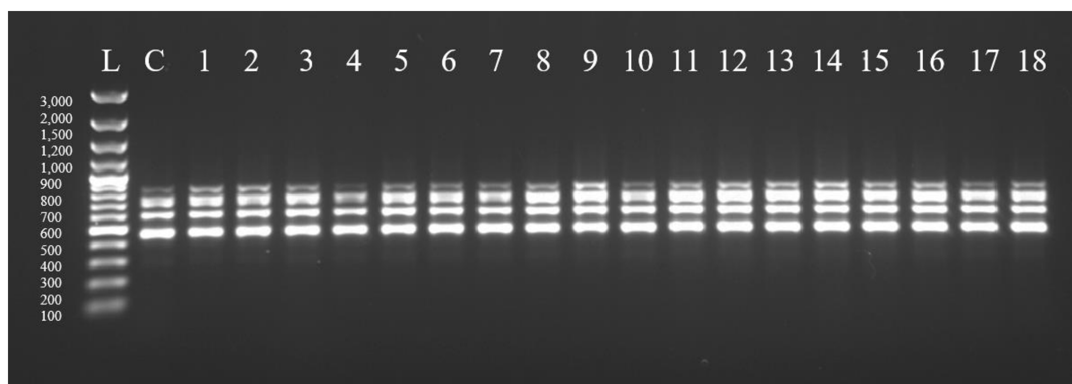

Figure 15. ISSR profile of control plant and *in vitro* regenerants of *Tacca integrifolia* using primer UBC 812; L – ladder, C – control plant, 1-18 *in vitro* regenerants (Author)

4.1.4 Analysis of ploidy level

In total, samples from 40 *in vitro* regenerants from medium supplemented by 1.0 mg. l^{-1} BAP and control sample of *T. integrifolia* were evaluated by flow cytometry. Two peaks are on histograms of relative nuclear content in all cases. The first peak represented nuclei in the G0/G1 phase of the cell cycle belonging to *Tacca integrifolia* sample and the second peak corresponded to nuclei of the internal standard (*Glycine max*) in the G0/G1 phase. The first histogram belongs to the control plant (Figure 16A) and the second histogram belongs to the *in vitro* plantlets regenerating from medium

with addition 1.0 mg. l^{-1} BAP (Figure 16B). Histograms clearly showed no changes in ploidy level.

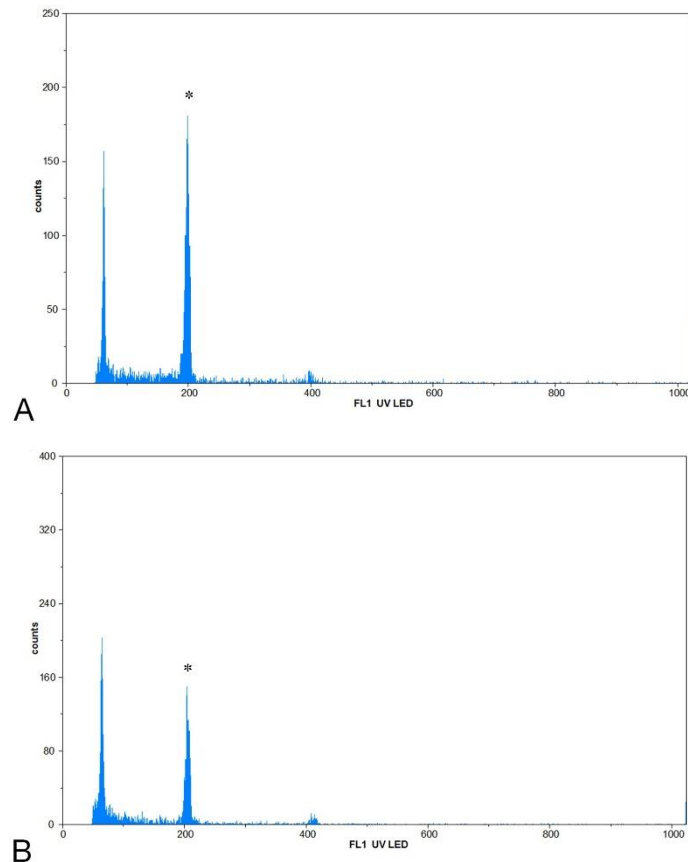

Figure 16. Flow cytometric histograms, peak marked as „*"corresponds to the internal reference standard *Glycine max*. A. Histogram of relative DNA content where peak is corresponding to the G0/G1 nuclei of control plant B. plant cultivated on medium supplemented with 1.0 mg. l^{-1} BAP

4.2 Polyploidization of *Tacca integrifolia*

Oryzalin and trifluralin were used in two concentrations 25µM and 35µM and two exposure time – 24 and 48 h for *in vitro* polyploidization. Results demonstrated that oryzalin was more efficient in polyploidization than trifluralin in all treatments. The highest percentage of chromosome duplication was observed in treatment with 25 µM of oryzalin and duration 48 h with efficiency 20%, which is two times higher than in other treatments of oryzalin. In this treatment, high survival rate was observed (95%). Overall, 12.5% of tetraploid plants and 95% survival rate were obtained through the induction of mitotic polyploidy using oryzalin treatment. Trifluralin had lower effect on

polyploidization than oryzalin, only 5% of polyploids were obtained from treatment with 25 µM per 24 h. Moreover, in case of trifluralin, negative effect on survival rate was observed. The lowest survival rate was in treatment with 25 µM of trifluralin and 48 h of exposure time (Table 6). Overall, 1.25% of tetraploid plants and 70% survival rate were measured through the induction of mitotic polyploidy using trifluralin treatment.

Table 6. Effect of oryzalin and trifluralin on polyploidy induction in *Tacca integrifolia*

	Treatment concentration (µM)	**Exposure time (h)**	**No. of shoots**	**Survival rate (%)**	**Tetraploid plants**	**Polyploidization efficiency (%)**
Control	0	0	20	100	0	0.0
Oryzalin	25	24	20	95	2	10.0
	25	48	20	95	4	20.0
	35	24	20	95	2	10.0
	35	48	20	95	2	10.0
Trifluralin	25	24	20	85	1	5.0
	25	48	20	50	0	0.0
	35	24	20	75	0	0.0
	35	48	20	70	0	0.0

After one month of cultivation on MS medium, plants affected by antimitotic agents started to regenerate from the initial explant. The process of regeneration was much slower than in control treatment, where plants started to regenerate one week after subculture. Slower growth, rooting and producing of adventitious shoots were also observed in tetraploid plants as well as in diploid plants affected by antimitotic agents. Each regenerated plant from polyploidization process was evaluated through flow cytometry.

In histograms, differences between diploids and tetraploids were detected (Figure 17). The DNA ratio in diploid ranged between 0.311-0.317 (Figure 17A), while in tetraploids, the DNA ratio range between 0.619-0.621 (Figure 17B). Differences in ratios between histograms proved successful induction of polyploidy.

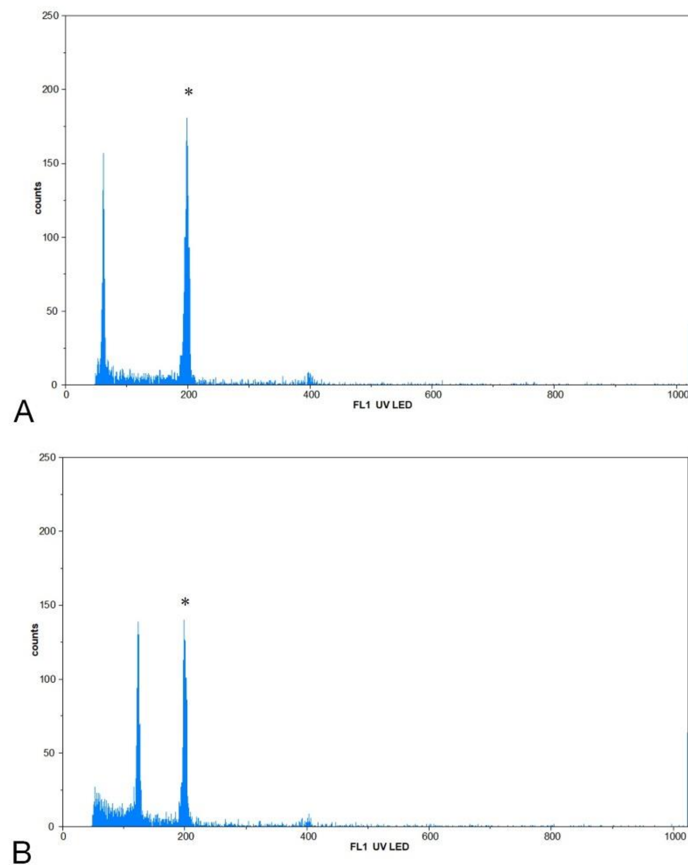

Figure 17. Flow cytometric histograms, peak marked as „*“corresponds to the internal reference standard *Glycine max*. A. Histogram of relative DNA content where peak is corresponding to the G0/G1 nuclei of diploid and B. tetraploid plant of *Tacca integrifolia* (author)

4.2.1 Growth and morphological characteristics of tetraploids

Primary study on evaluation of tetraploids was carried out. Nine tetraploid plants as well as nine control diploid plants were cultivated on the most appropriate medium optimized within this study (MS medium supplemented by 1.0 mg. l^{-1} BAP). Number of adventitious shoots, height of plant and number of leaves were measured after 8 weeks of cultivation on above mentioned medium.

Whole regenerating process was slower in tetraploid plants compared to diploids. Tetraploid plant showed supressed growth and lower number of shoots and leaves compared to diploids (Figure 18). However, the leaves were twisted and undeveloped, therefore tetraploid plants should be cultivated for longer time on suitable cultivation medium.

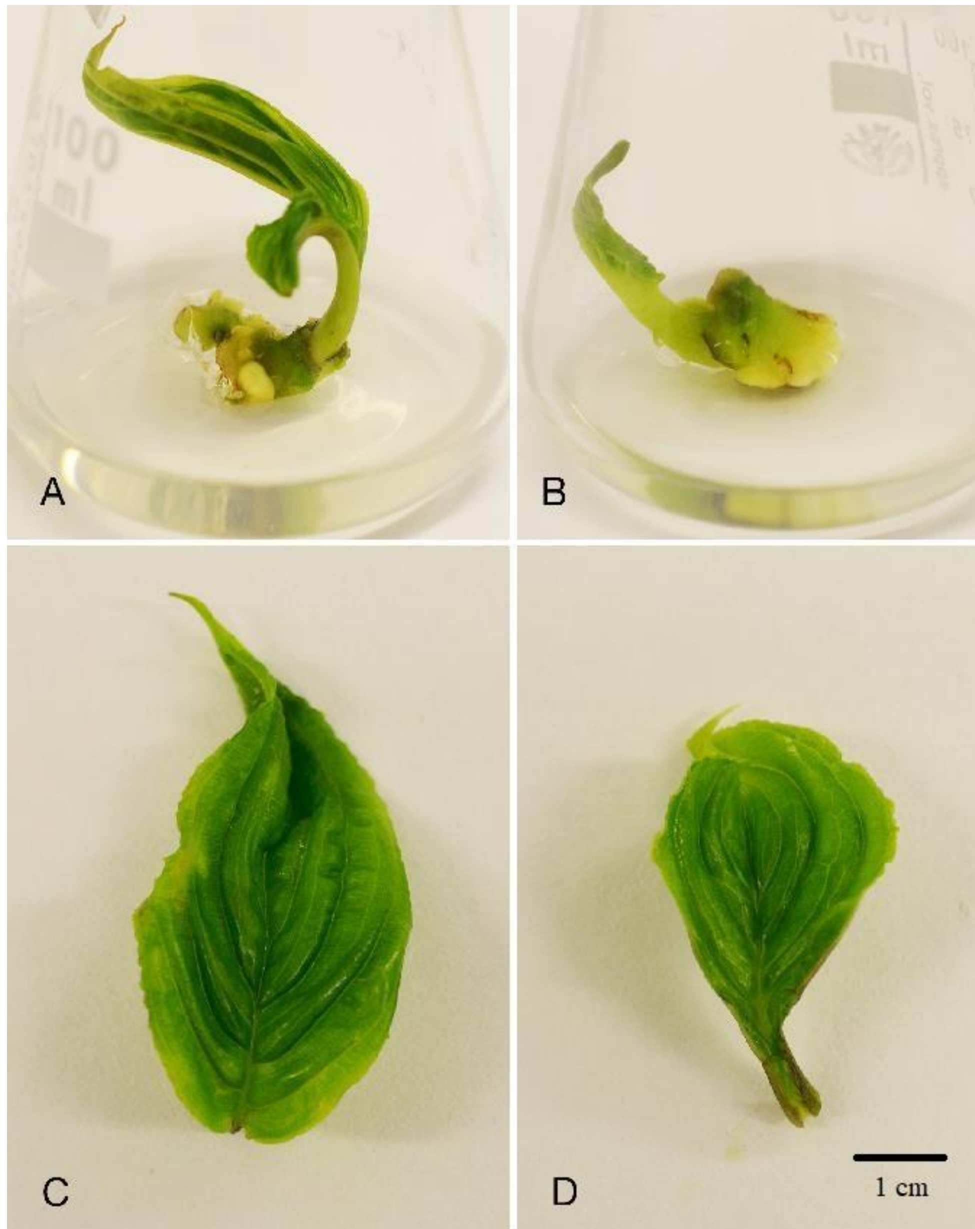

Figure 18. *In vitro* regenerants of *T. integrifolia* after two months of cultivation on MS medium with addition 1.0 mg. l^{-1} BAP. A. Diploid plant, B. Tetraploid plant, C. Leaf of diploid plant, D. leaf of tetraploid plant (Author)

Then, leaves of diploid and tetraploid plant were compared (length/width). Leaves of tetraploid was clearly shorter (35 mm) and narrower (25 mm) than leaves of diploid. Shape of leaves changed their character.

Thorough morphological evaluation of tetraploid plants in greenhouse can not be evaluated because lack of time.

5. Discussion

5.1 *In vitro* propagation

In vitro propagation technique is suitable to obtain a high amount of plant in short time (Engelmann 2011). This was confirmed in this thesis, where after 8 weeks of cultivation, *in vitro* plantlets of *T. integrifolia* were obtained originally from small shoots.

Several species from family Dioscoreaceae were also tested for micropropagation. Altough, *T. integrifolia* belongs to the same family as *Dioscorea* spp., different methods were used for micropropagation. While, small shoots were used as initial plant material in *T. integrifolia,* different parts of plant as axillary meristem (*D. deltoidea*; Furmanova et al. 1984), tubers (*D. opposite*; Jin xu et al. 2009) nodal segments (*D. opposite*; Shin et al. 2004), leaves, stem (*D. zingiberensis;* Shu et al. 2005) were used as initial material in *Dioscorea* spp.

Growth of plant is dependent on type of medium (Ponmurugan and Kumar 2011). In *T. integrifolia* the most suitable treatment, where plants produced the highest number of shoots, was MS medium with addition of 1.0 mg. l^{-1} BAP. This BAP concentration is much lower than in case of *T. chantrieri*, which produced the highest number of shoots on MS medium supplemented by 3 mg. l^{-1} BAP (Charoensub et al. 2008). However, in both studies MS medium without PGRs produced no or only few adventitious shoots. In *T. leontopetaloides*, BAP was successfully tested for shoot proliferation, however the most suitable concentration was much lower (0.1 mg. l^{-1}; Borokini et al. 2011) than in *T. integrifolia.* In following study focused on micropropagation of *Tacca leontopetaloides*, tested two different cytokinins BAP and kinetin were used; kinetin achieved better results in micropropagation (Martin et al. 2012). Another study about *T. leontopetaloides* compared even four types of cytokinins BAP, kinetin, zeatin and thidiazuron. In in this case, lower concentration of BAP and zeatin were used, however the highest concentration of BAP, e.g., 0.7 mg. l^{-1} BAP was the most successful for *in vitro* multiplication (Cepkova et al. 2015). In our study, *T. integrifolia* produced much more shoots on the most appropriate treatment in comparison to *T. leontopetaloides* and *T. chantrieri*. Successful application of BAP is reported in several studies focusing on family Dioscoreaceae (Das et al. 2013).

As a second cytokinin tested for *in vitro* propagation of *T. integrifolia*, was zeatin. While Cepkova et al. (2015) obtained better results of *T. leontopetaloides* on medium supplemented with zeatin than in treatments with BAP, *T. integrifolia* showed significantly better results in terms of multiplication using BAP.

Combination of auxins and cytokinins significantly increased shoot organogenesis in *T. leontopetaloides* (Borokini et al. 2011) and in *D. zingiberensis* (Chen et al. 2003) as well as in *D. hispida* (Behera et al. 2008) where combination of BAP and NAA was used. Nevertheless, unchanged effect had this combination on shoot organogenesis in *T. integrifolia*.

5.2 *In vitro* rooting

Several studies focused on species *Dioscorea hispida* (Behera et al. 2008), *D. opossitifolia* (Behera et al. 2009) and *D. esculenta* (Belarmini et al. 1991), related to the *Tacca integrifolia*, confirmed positive influence of NAA on superior production of roots. Although, higher concentration (2 mg. l^{-1}) of NAA was used in above mentioned species, in this thesis lower concentration (0.1 mg. l^{-1}) of NAA provided optimal results.

Explants of *T. integrifolia* produced only few roots on MS medium supplemented with cytokinins used for *in vitro* propagation, it is similar to results of Charoensub et al. (2008), where *T. chantrieri* did not produce any roots on MS medium supplemented with BAP nor with kinetin. NAA was used as the auxin promotion rooting as well as in study focused on *T. chantrieri* (Charoensub et al, 2008). *T. integrifolia* produced higher number of roots on lower NAA concentration (0.1 mg. l^{-1}) compared to the study of Charoensub et al. (2008), where the most efficient treatment was 0.3 mg. l^{-1}. Both plants produced roots on control treatments without auxins, however the production was lower. Martin et al. (2012) tested influence of IAA on *in vitro* rooting and higher concentration 0.5 mg. l^{-1} than in this study, was the most suitable. *T. leontopetaloides* also produced roots on higher concentration as 1 mg. l^{-1} and 2 mg. l^{-1}, while higher concentration of NAA was not suitable for *T. integrifolia*.

86.2% of surviving plants was observed after the *ex vitro* transfer of rooted explants of *T. integrifolia* which represents is lower efficiency than in study of Cepkova et al. (2015) in *T. leontopetaloides*. However, in both studies, plans after *ex vitro* transfer normally regenerated without morphological abnormalities.

5.3 Assessment of genetic fidelity of *in vitro* regenerants

Changes in DNA sequences or DNA ploidy level belong among the most common variation in the regenerants obtained via *in vitro* cultures (Brito et al. 2010). Therefore, genetic fidelity of *in vitro* regenerants of *T. integrifolia* was assessed using ISSR markers and flow cytometry.

Somaclonal variation is highly associated with indirect organogenesis – regeneration plants from callus (Karp 1995). Hence, direct organogenesis is preferred for massive production of true-to-type plant, because it maintain genetic stability (Ghimire et al. 2012). No somaclonal variation was detected in *Tacca integrifolia*, where the regenerated plants were obtained via direct organogenesis. Somaclonal variation was induced by regeneration from callus in *Smallanthus sonchifolius* and variation was detected using ISSR primers (Hammond et al. 2016) as well as genetic stability in *T. integrifolia.*

ISSR markers were used in *Tacca leontopetaloides* which is related to the *T. integrifolia*. The study of *T. leontopetaloides* provided information that micropropagation is suitable method for obtaining genetic uniform plants of this plant species, because no polymorphism among tested *in vitro* regenerants was obtained (Cepkova et al. 2015). These results are comparable to the results of our study focusing on *T. integrifolia*, where also no polymorphism was detected among tested *in vitro* plantlets.

In our study minimum 2 well visible and scorable bands were evaluated as well as in the study focusing on *T. leontopetaloides* (Cepkova et al. 2015). While study of *T. leontopetaloides* evaluated seven bands per one primer as the maximum with use of ISSR markers, in our study focusing on *T. integrifolia* four bands were obtain at maximum. In both studies two same primers UBC 809 and UBC 812 were used for ISSR analysis. However, the length of amplification fragments and number of scorable bands were lower in *T. integrifolia*. Same primers were used in the study focusing on ornamental *Puya berteroniana* for assessment of genetic stability. More visible bands were obtained from UBC 809 in *P. berteroniana* than in *T. integrifolia* and on the contrary more visible bands were obtained from primer UBC 812 in *T. integrifolia* than in *P. berteroniana*. Number of bands in individual primers are dependent on plant species, which is confirmed by study of *P. berteroniana* as well as this study of *T.*

integrifolia, because in comparison of two same bands, each of them is more appropriate for different plant (Viehmannova et al. 2016).

Use of ISSR markers for assessment of genetic stability were already tested in several ornamental plant species as *Gerbera* spp. (Bhatia et al. 2009), *Dendrobium nobile* (Bhattacharyya et al. 2014), *Lilium orientalis* (Liu & Yang 2012). Genetic fidelity was confirmed in all these plants.

Flow cytometry was used to detect any potential changes in the genome level in *T. integrifolia* and the results confirmed uniformity of *in vitro* regenerants without any variation in genome. Studies focusing on micropropagation of ornamental plant as *Chrysanthemum morifolium* (Naing et al. 2013) and *Puya beteroniana* (Viehmannova et al. 2016) proved no variation in ploidy level by flow cytometry.

5.4 Polyploidization

Two types of antimitotic agents were used for artificial mitotic polyploidization in *Tacca integrifolia.* The most appropriate treatment for polyploidization was 25 µM/48 h of oryzalin. However, longer exposure time of antimitotic agents were successfully used in several ornamental species as *Ranunculus asiaticus* (Dhooghe et al. 2009), *Spathiphyllum wallisii* (Eeckhaut et al. 2004) *Tulipa gesneriana* (Chauvin et al. 2005). Then, much higher concentration in short exposure time were used in ornamental species as *Watsonia lepida* (120 µM/24 h of oryzalin; Ascough et al. 2008), or *Alocasia* (289 µM/ 24 h od oryzalin; Thao et al. 2003).

The largest number of tetraploids was obtained from treatment 25 µM per 48 h (20% of polyploids) which is in agreement with study focusing on polyploidization in *Smallanthus sonchifolius* (Viehmannova et al. 2009). On the contrary induction of mitotic polyploidization using trifluralin was also tested in *Rosa* spp., where plants successfully doubled number of chromosome in similar concentration (24 µM per 24 and 48 h of exposure time) and 37.5% and 62.5% of polyploid plants were obtained (Khosravi et al. 2008) – these results are more efficient than even results of oryzalin in *T. integrifolia.* The survival rate of plants affecting by trifluralin was 70% in *T. integrifolia*, which is much higher compared to 40% survival rate of *Rosa* spp. (Khosravi et al. 2008).

Totally, plants, treated with oryzalin achieved superior results than trifluralin which was also described by Denaeghel et al. (2015) in *Escallonia* spp. Higher survival rate in mitotic polyploidization by oryzalin of *T. integrifolia* than trifluralin is corresponding to the study focusing on *Rosa* spp. (Khosravi et al. 2008) where were used same antimitotic agents.

Induced polyploidy could change the phenotypical character, which was also proved in primary study of mitotic polyploidization in *T. integrifolia*. Regenerated *in vitro* plantlets were smaller than control plant as well as in study about *Hibiscus* (Contreras et al. 2009). On the contrary in case of *Anemone sylvestris*, plants increased their habit (Zahumenicka et al. 2017). Tetraploid plants of *T. integrifolia* changed shape of leaves, which are now more orbicular than obovate. Similar change of leaves was reported in *Alocasia* 'Green Velvet' (Thao et al. 2002).

6. Conclusion

In this study, an efficient protocol for micropropagation of *Tacca integrifolia* was developed. It was evaluated that application of cytokinins increases the production of adventitious shoots and the use of auxins lead to root development in *T. integrifolia*. The most efficient treatment for *in vitro* propagation was MS containing 1.0 mg. l^{-1} BAP. On the contrary, zeatin did not prove to be an appropriate type of cytokinin for *in vitro* propagation. For *in vitro* rooting, auxin NAA was efficient for increased production of roots. The most suitable treatment was MS medium supplemented by 0.1 mg. l^{-1} NAA. After *ex vitro* transfer and acclimatization of plants, 86% survival rate of plants was obtained. No somaclonal variation was detected by ISSR markers and flow cytometry.

This thesis is the first scientific report focused on *in vitro* induction of mitotic polyploidization of *T. integrifolia*. Mitotic polyploidization was successfully induced using oryzalin and trifluralin. Plants exposed to 25 µM of oryzalin for 48 h achieved the highest (20%) production of tetraploid plants. Primary study focusing on evaluation of morphological characteristics of teraploid plants showed influence of chromosome doubling on changing size and shape of leaves as well as size of plant.

In conclusion, both techniques, micropropagation and polyploidization were optimized in *T. integrifolia*. Micropropagation can be used for mass commercial production of genetic uniform plants as well as for conservation purposes of *T. integrifolia*, whereas polyploidization may be considered as a suitable technique for breeding programmes to increase commercial potential of *T. integrifolia*.

7. Recommendation

Protocol for micropropagation and polyploidization of *Tacca integrifolia* was optimized. Within further research, ornamental potential as well as physiological aspects of tetraploid plant cultivation should be tested. Beside ornamental uses, *T. integrifolia* is also a medicinal plant. Therefore, the study of secondary metabolites in tetraploid plants would be of high interest.

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