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Use of *in vitro* cultures in *Tacca integrifolia*

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I confirm by my signature, that this bachelor's thesis "Use of *in vitro* cultures in *Tacca integrifolia*" is my original work, where I am using cited sources, which are written in the end of the thesis.

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Abstract

Tacca integrifolia (Disocoreaceae) is a perennial ornamental and medicinal plant originating from tropical Asian regions. The objective of this thesis was an optimization of micropropagation in this species. Shoot proliferation was tested on MS medium (Murashige and Skoog, 1962) with different concentrations of plant growth regulators (PGRs). Zeatin and 6-benzylaminopurine (BAP) were used at concentrations 0.3-1.5 $mg.l^{-1}$. In total, 9 treatments including control were tested. For *in vitro* rooting, α – naphthaleneacetic acid at concentrations 0.1 and 0.3 $mg.l^{-1}$ was tested. The most effective medium for shoot proliferation was medium supplemented with 1.5 $mg.l^{-1}$ BAP, providing 2.89 shoots per explant. The highest number of roots was achieved on medium with addition of 0.3 $mg.l^{-1}$ NAA (3.40 roots per explant) with length 0.83 cm. Well-rooted plants were successfully transferred to the green house. The percentage of survival plants was 100%. Optimized protocol of micropropagation might be used as an effective tool to obtain sufficient number of genetically uniform plants of *T. integrifolia* in short time, for ornamental, medicinal and conservation purposes.

Keywords: Adventitious shoots · Dioscoreaceae · *in vitro* propagation · plant growth regulator · rooting · *Tacca integrifolia*

Abstrakt

Tacca integrifolia (*Dioscoreaceae*) je vytrvalá rostlina z tropických oblastí Asie. Tato rostlina je využívána pro okrasné a léčivé účely. Cílem této práce bylo optimalizovat proces mikropropagace u tohoto druhu. Tvorba odnoží byla testována na MS (Murashige and Skoog, 1962) médiu s přídavkem různých koncentrací růstových regulátorů. Pro odnožování byly použity cytokininy BAP a zeatin v koncentracích 0,3-1,5 $mg.l^{-1}$. Celkově bylo testováno 9 médií včetně kontrolní varianty. Pro *in vitro* zakořeňování byl využíván auxin NAA v koncentracích 0,1 a 0,3 $mg.l^{-1}$. Pro tvorbu odnoží bylo optimální medium s přídavkem 1,5 $mg.l^{-1}$ BAP, na kterém rostliny vytvářely v průměru 2,89 nových odnoží. Nejefektivnější medium pro tvorbu kořenů bylo médium s 0,3 $mg.l^{-1}$ NAA, kde se vytvářelo průměrně 3,40 kořenů na rostlinu, dlouhých 0,83 cm. Dostatečně zakořeněné rostliny byly úspěšně převedeny *ex vitro*, do skleníku. Úspěšnost převodu byla 100%. Protokol optimalizovaný v této studii lze využít pro efektivní množení rostlin okrasného druhu *T. integrifolia* při zachování genetické uniformity regenerantů. Tento protocol lze rovněž využít pro namnožení rostlin pro účely uchování rostlinného materiálu..

Klíčová slova: Adventivní výhony · *Dioscoreaceae* · *in vitro* množení · rostlinné růstové regulátory · *Tacca integrifolia* · zakořeňování

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List of abbreviations

ANOVA	analysis of variance
BAP	6-benzylaminopurine
CULS	Czech University of Life Sciences Prague
FTA	Faculty of Tropical AgriSciences
MS	Murashige and Skoog medium (1962)
NAA	α – naphthaleneacetic acid
PGR	plant growth regulator
TDZ	thidiazuron

1 Introduction

Tacca integrifolia, known as white bat flower, is a species of the family Dioscoreaceae. It is mainly distributed in tropical Asian regions (Caddick *et al*., 2002). It is a short-stemmed perennial ornamental plant (Chee *et al*., 2013). It is expressive plant, which can grow up to 1 m in height. It has got unusual whisker-like bracts below the flowers, which hang down as long as 30 cm (Chee *et al*., 2013). The plant can be used as interior plant because of its showy appearance. According to Plants rescue (2016) it is also possible to use it in outdoors. *Tacca integrifolia* is also a medicinal plant which is used in China for treatment of gastric ulcer, enteritis and hepatitis (Dictionary of Chinese Medicinal Materials, 1977) and in Thailand it is used for controlling of blood pressure and improving sexual function (Chuakul *et al*., 2000).

In nature, *Tacca integrifolia* is propagated by stem buddings and by seeds (Mohd Fuat *et al*., 2007). However generative propagation is rather difficult and slow, because in forests can be found only a few seeds (Charoensub *et al.,* 2008). Micropropagation is the most efficient method which enables to obtain a big amount of genetically uniform plants.

Thus, the objective of this thesis was to develop a suitable protocol for micropropagation of *Tacca integrifolia*. The micropropagation can be used for highly effective production of this ornamental and medicinal plant and also for conservation of plant material.

2 Literature review

2.1 *Tacca integrifolia*

2.1.1 Taxonomy and relative species

The families constituting order Dioscoreales are: Dioscoreaceae (including former Taccaceae and Trichopodaceae), Burmanniaceae (including Thismiaceae) and Nartheciaceae (Caddick *et al*., 2002). Dioscoreaceae now contain four district genera, *Dioscorea*, *Stenomeris*, *Tacca* and *Trichopus*. The Malagasy endemic *Avetra sempervirens* is closely related to *Trichopus zeylanicus* and is reclassified as a second species of this genus. Some previous publications (Knuth, 1924; Burkill, 1960) also placed *Avetra*, *Stenomeris* and *Trichopus* in Dioscoreaceae, but most others have treated them as separate genera of Dioscoreales: *Avetra* and *Trichopus* in Trichopodaceae and *Stenomeris* in Stenomeridaceae (Hutchinson, 1959; Takhtajan, 1987; Huber, 1991).

The pantropical family Dioscoreaceae comprises approximately 650 species (Govaerts *et al*., 2007). Taxonomic revisions and studies of species boundaries have been rare. As much several taxonomic assemblages in the family can be identified but without specific delimitation. Pedralli (1998) and Couto (2010) thus indicating the necessity for more research focusing on the family.

Tacca integrifolia commonly known as white bat flower, is a species of flowering plant from family Dioscoreaceae (Caddick *et al.*, 2002). Originally, the genus was included in the Taccaceae (Dumortier, 1829). *Tacca* differs from other Dioscoreaceae in its acaulescent habit and unilocular ovaries with parietal placentation, but it shares numerous characters with this group including tuberous underground parts rich in steroidal saponins, petiolate, reticulate-veined leaves, and reflexed stamens. In vegetative morphology the entire-leaved species of *Tacca* (*T. chantrieri* and *T. integrifolia*) closely resemble *Trichopus zeylanicus*, and both genera are also characterised by a papillose, umbrella-shaped style. The unusual pseudoumbellate inflorescence, which led Drenth (1972) to associate *Tacca* with Amaryllidaceae, has been reinterpreted as cymose (Dahlgren *et al*., 1985). This classification recognises the close relationship between *Dioscorea*, *Stenomeris*, *Tacca*

and *Trichopus* relative to other Dioscoreales (Burmanniaceae and Nartheciaceae) by uniting them into a single family (Caddick *et al*., 2002). With exception of *Tacca*, all these taxa have been included as genera of Dioscoreaceae in previous publications. These taxa have 15 species of plants (Knuth, 1924; Burkill, 1960; Dahlgren *et al*., 1985).

2.1.2 Origin and geographical distribution

Overall, the geographical origin of the *Tacca* genus is unknown, but a centre of recent speciation is the Southeast Asia (Drenth, 1972). It is possible to find it in tropics of Cancer and Capricorn (Drenth, 1976).

From this area 9 species are known. The 4 entire-leaved species occur in the area covered by East India, Bangladesh, Thailand, South China, Indo-China, the Malay Peninsula, Sumatra, Borneo and West Java. The 4 palmate-leaved species occur in the area covered by Indo-China, Malesia, the Solomons and the Caroline Islands. One species is in tropical South America (Drenth, 1976). *Tacca leontopetaloides* has a colossal distribution from the west coast of Africa to Easter Island in the east Pacific. *Tacca parkeri* is from tropical South America. The supposed affinities between species and the pantropical distribution point to an old origin of the family (Drenth, 1972) (Figure 1).

Tacca integrifolia is predominantely distributed in tropical regions of Asia (Caddick *et al.*, 2002) and one of the rare species in Tibet (Dong *et al.*, 2007). It is primarily paleotropical in distribution and its current distribution centre is also Southeast Asia.

Figure 1. Distribution of *Tacca* species (Drenth, 1976)

2.1.3 Ecology

Tacca integrifolia is a plant, which is growing up from sea level up to 1200 m, rarely to 1500 m altitude. It is proved that plants from the hills could be larger than others from the plains (Drenth, 1972). In many cases *Tacca integrifolia* occur in evergreen moist primary and secondary forests, e.g. on steep slopes, ridges, or near water, sometimes on roadsides and in clearings (Drenth, 1972). It is growing on various fertile soils e.g. sandy or stony substrata, limestone or red earth and it need shade from the intense afternoon rays (Drenth, 1972; Meerow, 1995). The suitable substrate is composed of 50% pine bark, 40% peat and 10% sand, this medium supports rapid growth (Meerow, 1995). The plant need high temperatures and it is necessary to protect plant from cold, because its leaves can be burned by cold (Meerow, 1995).

2.1.4 Morphology

2.1.4.1 Habit

Tacca integrifolia is a terrestrial, long-lived plant (Drenth, 1972). It is a plant, which can grow to the 100 cm. *Tacca integrifolia* is a short-stemmed tuberous plant (Chee *et al*., 2013).

2.1.4.2 Foliage

Tacca integrifolia has big entire leaves, which grow up from the top of rhizome (Meerow, 1995; Chee *et al*., 2013). Leaves are polished, ovate and dark green. They are about 25 cm wide and to 60 cm long (Seedaholic, 2016). Leaves are similar to leaves of *Spathiphyllum* (Meerow, 1995). The leaf blade is downcast and the petiole has a sheathing base. Plants have a reticulate venation (Drenth, 1972) (Figure 2).

Figure 2. Foliage of *Tacca integrifolia* (Plantguide, 2013)

2.1.4.3 Inflorescence

It is possible to partition species of genus *Tacca* into three groups, every group has a different inflorescences. I. inconspicuous inflorescences with small bracts and short bracteoles, e.g. *Tacca leontopetaloides*, *Tacca plantaginea*, *Tacca parkeri*; II. inconspicuous inflorescences only with bracts, it have flowers like *T. palmata*, *T. palmatifida*; III. very conspicuous inflorescences with large bracts and long bracteoles, as *Tacca ampliplacenta*, *Tacca integrifolia*, *Tacca chantrieri, Tacca subflabellata* (Zhang *et al*., 2011).

Tacca integrifolia has strange whisker-like bracts approximately 30 cm long (Drenth, 1972; Chee *et al.*, 2013). Cluster of dark violet flowers and white bracts is the reason, why the second name of *Tacca integrifolia* is white bat flower. Several broad and bracts look like a bat wings (Meerow, 1995). Flowers of genus *Tacca* have usually the same structure (Drenth, 1972). The six tepals are growing together or they are pulpy in some species. Stamens are basally connected to the tepals (Caddick *et al.*, 2008). *Tacca integrifolia* has a dark violet, epigymous and bisexual flower (Drenth, 1972; Chee *et al*., 2013). Fruits of *T. integrifolia* are fleshy, they are perspicuous on the first view. Fruits are from green to black (Drenth, 1972; Chee *et al.*, 2013) (Figure 3).

Figure 3. Inflorescence of *Tacca integrifolia* (Block botanical gardens, 2014)

2.1.4.4 Rhizomes

The rhizomes of genus *Tacca* are divided into three types: I. a vertical elongated rhizomes with an apical growth, II. roundish rhizomes with an apical cavity showing the growth centre, III. horizontal elongated rhizomes (Drenth, 1972).

Tacca integrifolia is a plant with cylindric rhizome (Kitjaroennirut *et al.*, 2005). Its rhizome grows vertically (Meerow, 1995).

2.1.5 Uses and properties

Tacca integrifolia is an ornamental plant (Charoensub *et al.*, 2008. It is possible to cultivate *T. integrifolia* like indoor flower or in outdoors arrangements (Plants rescue, 2016). However, this exotic flower is not suitable as a cut–flower, because after cutting, the flower wilt soon, even if is placed in the water (Meerow, 1995).

Tacca integrifolia has been used in ethnobotanics. Especially, in China has been used as treatment for gastric ulcer, enteritis and hepatitis (Su, 1997). In Thai herbal medicine, rhizomes of *Tacca integrifolia* are used for improving sexual function and for control of blood pressure, it depends on methanol extract, which *Tacca integrifolia* contains (Wutthithamawet, 1997; Chuakul *et al*., 2000; Kitjaroennirut *et al*., 2005).

Cooked rhizomes are usually used as aphrodisiac for men and as treatment to irregular menses for women. Pulp of rhizomes is good to treat skin rashes by insect, wounds, swellings, sores, snakebites, rheumatism. Cooking whole plant is to treat diabetes, haemorrhoid and kidney problems (Chee *et al*., 2013).

2.2 Reproductive biology of *Tacca* genus

Tacca genus has unusual reproductive biology with a lot of uncertainties. Research focused on biological characters in the genus *Tacca,* are very rare. There is a little information about pollination, mating system, biosystematics, reproductive biology and conservation biology. There are available some observations and speculations (Faegri and Van der Pijl, 1971; Drenth, 1972; Saw, 1993; Lim, 2011).

Tacca is predominantely cross-pollinated plant (Faegri and Van der Pijl, 1966). It is possible that dark floral colours and big bracts relate with fly pollination. In genus *Tacca* exists a "deceit syndrome". The syndrome means that *Tacca* species have reproductive structures, similar to decaying organic material (Endress, 1995; Stevenson, 2004; Zhang *et al*., 2007). The impression of decaying organic material attract group of *Diptera* to blossoms in spite of dark colours of flower, which are not attracted under normal circumstances for this class of pollinators. It changes to positive when it is in the presence of odour decaying protein (Drenth, 1972).

Tacca species have no nectar and produce a small amount of pollen. Pollinators are attracted only for a "deceit syndrome" and then they are facilitating a cross-pollination (Zhang *et al*., 2005, 2007).

These observations of *Tacca* genus disagree with a preconditions of showy inflorescence structure. It is paradox because *Tacca* genus has interesting inflorescence but with no interesting function for pollinators (Zhang *et al*., 2005).

2.3 Plant husbandry

2.3.1 Cultivation

It is possible to cultivate *Tacca integrifolia* indoors. *T. integrifolia* grows well in the same conditions like orchids and many epiphytic formes with a lot of humidity,

strong airflow, and moderate light and temperatures. When *Tacca integrifolia* grows indoors, it needs bright light, but it does not like direct sunlight. The suitable temperature is between 21-32°C and good air-circulation, it is beneficial for tropical plant. These plant naturally grow in the tropical rain forests with high temperature and with a lot of vapour during the year. Humidity above 50% is optimal for these plants, but it is difficult to ensure this humidity in homes, it is possible to elevate humidity by a humidifiers. During the growing season (late spring to late summer) plant needs periodical watering. On the contrary, during the rest of growing season should be potting only a bit moist. It is more preferable to use rainwater than tap water, because of chlorine and fluoride and higher concentrations of salt in tap water. Tap water is not suitable for plant and leaves because of their leaves could become brown. When *T. integrifolia* is cultivated on the gardens, should grow in the soil only in tropical months, in other month it grow in the pot (Plants rescue, 2016).

2.3.2 Propagation and planting

In cultivation, *Tacca integrifolia* is a self-pollinate plant (Meerow, 1995). It is possible to propagate *T. integrifolia* from seed or stem budding (Mohd Fuat *et al*., 2007). The seeds should be cleaned of the pulp and should be dried and sown immediately (Houseplant about, 2015). These seeds should be sown to germination substrate with the same amount of peat and perlite, or vermiculite and perlite. Seeds need warm, moist and bright light for a good propagation. They will germinate about 8-12 weeks. When seedlings have a first leaf well- developed, it is possible to put them to a small pots (Meerow, 1995). When plants are about 5 cm high, it is possible to move them into bigger pots (Plants rescue, 2016).

Tacca can be propagated also vegetatively, by offsets, which is growing from the sides of the base. The offsets should be placed individually in pots, should be drained and should be there a same amount of compost, peat and coarse sand. The offsets need a temperature of 21 °C and a few of moist. They will be well-rooted for a few weeks, after it is possible to put the plants to more light and air (Plants rescue, 2016).

2.3.3 Fertilization

Tacca integrifolia does not like strong fertilizers. It is possible to use commercial plant fertilizers and organic compost (Plants rescue, 2016). *Tacca* needs a biweek fertilization with a soluble orchid fertilizer or with a slow soluble fertilizer (University of Florida, 2016) during the growing season (Houseplant about, 2015), but it is not good to use fertilizers during the rest period (Plants rescue, 2016).

Regular fertilization is also beneficial for seedlings. When they have a few of fertilizer, the can have got a chronic chlorosis (Meerow, 1995).

2.3.4 Pest and diseases

One of the benefits of white bat flower is its rezistence against plan pest (Nuanla and Sruamsiri, 2000) and also against diseases (Meerow, 1995). Possible problems are salts in fertilizers, which can lead to plant stress and its root can be burned, also the plant can be dehydrate (Plants rescue, 2016). On the other hand for example, root disease can start also in *Tacca integrifolia,* which will be consistently wet (Logees, 2016).

2.4 Use of *in vitro* technologies

2.4.1 Use of *in vitro* technologies in Dioscoreacae

In vitro cultures in family Dioscoreaceae, where the genus *Tacca* belongs, are very often used for micropropagation. There are available many studies on micropropagation in this family.

Classical methods of propagation yams (*Dioscorea*) by seeds and by planting yams are slow and are not advantageous for quickly proliferation of pathogens. To overcome these problems, *in vitro* propagation of plant material, via nodal segment culture, indirect organogenesis and direct or indirect somatic embryogenesis (Mantell *et a.l*, 1978; Ng, 1992; Twyford and Mantell, 1996) can be used. *In vitro* techniques has been used for many *Disocorea* species like *D. composite* Hemsl, D. *cayenensis* (Viana and Mantell, 1989*) D. rotundata* (Balogun *et al.*, 2006), *D. nipponica* Makino (Chen *et al*., 2007) and the *D. cayenensis – D. rotundata* complex (Ovono *et al* 2007, 2009,

2010a). Many studies are focused on medicinal yams such as *D. floribunda* (Chatuverdi *et al.*, 1982), and *D. deltoidea* (Grewal *et al.*, 1977).

In vitro cultured shoots of some *Dioscorea* species produce microtubers in special conditions and could have a perfect potential for quick multiplication and distribution of healthy clonal material in international yam germplasm exchange programmes. Numerous factors participate on growth of microtubers in *Dioscorea* such as plant growth regulators, sucrose concentration or photoperiod (Mantell and Hugo, 1989; Jean and Cappadocia, 1992; Ng, 1992).

2.4.2 Use of *in vitro* technologies in *Tacca* genus

For the *Tacca* genus, studies on shoot organogenesis for two species *T. chantrieri* (Charoensub *et al*., 2008) and *T. leontopetaloides* (Borokini *et al*., 2011) are available. These primary studies detected response of *Tacca* species to *in vitro* cultures and provided an important data for further research (Cepková *et al*., 2015) focused on micropropagation of *T. leontopetaloides*.

Tacca leontopetaloides can be propagated by seeds, but the seeds have a low germination capacity. According to horticultural tradition, plants are propagated by tuber or shoot (Spennemann, 1994; Borokini *et al*., 2011), but this technique also cannot produce a massive amount of plants. The most suitable techniques are *in vitro* cultures for the possible rapid multiplication and production of large amount of plantlets in a short time (Engelmann, 2011).

According to Cepková *et al.* (2015) from four cytokinins (BAP, zeatin, kinetin and thidiazuron (TDZ)) tested, zeatin has got the highest effect on shoot organogenesis in *T. leontopetaloides*. Previously, Borokini *et al.* (2011) recommended BAP for *in vitro* propagation of the same species, however in this study, effect of various cytokinins was not investigated.

Tacca chantrieri (Bat flower), another species of the *Tacca* genus, where seeds have very slow germination capacity*, in vitro* cultures are being also used for propagation of plants (Charoensub *et al*., 2008).

In *T. integrifolia*, process of *in vitro* propagation has not been optimized yet. Optimization of micropropagation protocol could enable to produce numerous plants for ornamental and medicinal purposes (Sulong, 2013).

3 Objectives of the thesis

The main objective of this thesis was to develop a suitable protocol for micropropagation of *Tacca integrifolia*.

The main objective can be divided into three following parts:

- Optimization of *in vitro* plant propagation
- Optimization of *in vitro* rooting
- Transfer of regenerated plants *ex vitro*

Optimization of all phases of micropropagation in the species *Tacca integrifolia* would increase the efficiency of plant propagation of this ornamental and medicinal species.

The objectives were determined based on two hypotheses:

- Cytokinin induces production of adventitious shoots.
- Auxin induces production of roots and their length.

4 Material and methods

4.1 Plant material

Seeds of *Tacca integrifolia* were obtained via Index Seminum from Universitatea Bades–Bolyai Gradina Botanica „Alexandru Borza“ in Romania in 2013. *In vitro* culture was established within previous research carried out in the Laboratory of Plant Tissue Cultures, FTA.

For the experiment within this thesis, as an initial plant material, *in vitro* plants of *Tacca integrifolia* were used.

4.2 Methods

4.2.1 *In vitro* propagation of plant material for experiment establishment

Firstly, it was necessary to ensure sufficient plant material for further experiment. Plants of *T. integrifolia* were cultivated on MS medium (Murashige and Skoog, 1962) with 0.5 $mg.l^{-1}$ BAP. Since *in vitro* plants did not produce sufficient number of shoots on this medium, the plants were transplanted on MS medium with higher concentration of BAP, i.e. 0.7 $mg.l^{-1}$. Plants were repeatedly sub-cultured on the same medium every 3-4 weeks. Plants were cultivated for 28 days at 25/23°C under 16 hours of light and 8 hours dark regime with 36 $\mu mol\ m^{-2}.s^{-1}$ fluorescent light.

4.2.2 *In vitro* propagation of *T. integrifolia*

To standardize the experiment on *in vitro* propagation of plants, the plants of the same size with cutted leaves and no adventitious shoots were used (Figure 4). For experiment, MS medium supplemented with 100 $mg.l^{-1}$ *myo*-inositol, 30 $g.l^{-1}$ sucrose, 8 $g.l^{-1}$ agar and with different types and concentrations of plant growth regulators (PGRs) was used. pH of the medium was adjusted to 5.7 and it was autoclaved in 121°C in 1.1 atm, for 20 min. Each PGR was used individually. Experiment consisted of one control treatment (without PGRs), four treatments with concentrations of BAP (0.3 $mg.l^{-1}$ to 1.5 $mg.l^{-1}$), and four with concentrations of zeatin, (0.3 $mg.l^{-1}$ to 1.5 $mg.l^{-1}$) (Table 1). In

each treatment, ten repetitions were used.

Table 1. Various treatments for *in vitro* propagation of *Tacca integrifolia*

Treatment	BAP ($mg.l^{-1}$)	zeatin ($mg.l^{-1}$)
1.	0.3	
2.	0.7	
3.	1.0	
4.	1.5	
5.		0.3
6.		0.7
7.		1.0
8.		1.5
Control	0	0

Figure 4. Initial explant used for the experiments (author)

4.2.3 *In vitro* rooting and *ex vitro* transfer

To induce rooting, shoots produced within a previous experiment were placed on MS medium with auxin NAA at concentrations of 0.1 $mg.l^{-1}$ and 0.3 $mg.l^{-1}$. The cultures were cultivated 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. After 4 weeks, number of roots and length of roots were measured.

For *ex vitro* transfer, well-rooted plants were chosen. First, roots were thoroughly washed from medium under tap water. Thereafter, plants were planted to substrate consisting of perlite-garden soil in ratio 1:1. The plants were covered with a plastic cover and the cover was gradually removed (during three weeks). The survival rate was evaluated 3 weeks after *ex vitro* transfer.

4.2.4 Statistical evaluation

All results were evaluated by the statistical analysis. Statistical analysis of data consisted of analysis of variance (ANOVA) and the significantly different means were identified using the Tukey´ss HSD test (p = 0.05) [StatSoft STATISTICA 12.0].

5 Results

5.1 Production of adventitious shoots

Production of adventitious shoots was tested on MS medium supplemented with two different cytokinins BAP and zeatin at concentrations 0.3-1.5 mg.l^{-1}.

After 28 days, the highest number of shoots was obtained on medium supplemented with BAP at the highest concentration tested, i.e., 1.5 mg.l^{-1} (2.89 shoots per explant) (Figure 5A). Also callus was formed from 0.7 mg.l^{-1} concentration of BAP. In lower concentrations of BAP the number of shoots was decreased (Table 2). On medium without BAP, plants produce only 0.13 shoots per plant (Figure 5B). Also, the height of plant was increased with increasing of concentration of BAP. Overall, medium supplemented with BAP produced more shoots, but the highest was lower than in medium supplemented with zeatin.

Plants, which were cultivated on medium supplemented with zeatin produced less of adventitious shoots. Neither in the highest concentration of zeatin, plants produced such a big amount of shoot as in medium with BAP. The medium with the highest concentration (1.5 mg.l^{-1}) of zeatin produced almost the same amount of number of new shoots (1.60 shoots per explant) as in medium with the lowest concentration (0.3 mg.l^{-1}) of BAP (1.40 per explant). On the other hand, plants cultivated on medium with zeatin were higher and had bigger and wider leaves than on medium with BAP (data not shown). From the zeatin concentrations the most efficient was with concentration 0.7 mg.l^{-1}. The highest average height of plant was 12.1 cm. The lowest plants were obtain on the medium with 0.3 mg.l^{-1} BAP with average height 8.25±0.47 cm of plant (Table 2).

Plants cultivated on control medium reached comparable height as plants cultivated on medium with cytokinins, however, number of newly developed shoots was very low (Figure 5A, Table 3).

Table 2. Results of *in vitro* propagation of *Tacca integrifolia*

Treatment	BAP ($mg.l^{-1}$)	zeatin ($mg.l^{-1}$)	Number of shoots	Height of plant (cm)
1.	0.3		1.40±0.37 abc	8.25±0.47c
2.	0.7		1.30±0.50 abc	8.88±0.50 bc
3.	1.0		2.44±0.44 ab	8.97±0.39 bc
4.	1.5		2.89±0.63 a	9.94±0.54 abc
5.		0.3	0.56±0.18 bc	8.67±0.85 c
6.		0.7	0.60±0.22 bc	12.10±0.38 a
7.		1.0	1.11±0.35 abc	11.71±0.72 ab
8.		1.5	1.60±0.54 abc	10.70±0.97 abc
Control	0	0	0.13±0.13 c	9.81±0.73 abc

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

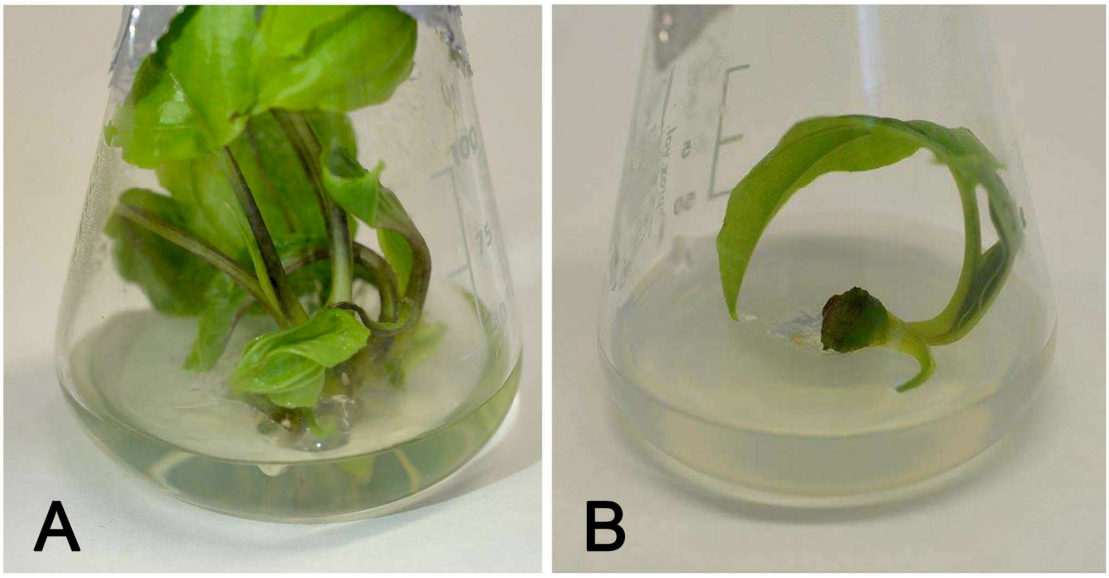

Figure 5. Explants on medium supplemented with 1.5 $mg.l^{-1}$ BAP and free PGRs control medium (author)

5.2 *In vitro* rooting and *ex vitro* transfer

Plants cultivated on MS medium with cytokinins produced only very low number of roots, the roots were extremely short, and they did not enable *ex vitro* transfer of the plants (Table 3). Medium without PGRs produced comparable number of roots as medium with addition of cytokinins. Therefore, induction of roots was tested on media containing 0.1 and 0.3 $mg.l^{-1}$ NAA. For number of roots, the most efficient was MS medium supplemented with 0.3 $mg.l^{-1}$ NAA. The average number of roots was 3.40 and their length was 0.83 cm and the percentage of rooting was 100%.

However for length of roots was more suitable MS medium supplemented with concentration 0.1 $mg.l^{-1}$ NAA. The average of length of roots was 1.12 cm (Table 3).

Well-rooted plants were transferred to the green house (Figure 6) The survival rate 3 weeks after *ex vitro* transfer was 100%.

Table 3. Results of *in vitro* rooting of *Tacca integrifolia*

Treatments	BAP $mg.l^{-1}$	zeatin $mg.l^{-1}$	NAA $mg.l^{-1}$	Number of roots	Length of roots (cm)
1.	0.3			1.50±0.27 bc	0.71±0.17 ab
2.	0.7			1.90±0.48 abc	0.71±0.11 ab
3.	1.0			1.22±0.22 bc	0.29±0.10 b
4.	1.5			1.78±0.49 abc	0.84±0.13 ab
5.		0.3		0.78±0.36 bc	0.47±0.14 ab
6.		0.7		0.90±0.28 bc	0.41±0.10 ab
7.		1.0		0.67±0.24 bc	0.65±0.30 ab
8.		1.5		0.40±0.16 c	0.30±0.07 ab
9.			0.1	2.40±0.56 ab	1.12±0.14 a
10.			0.3	3.40±0.56 a	0.83±0.14 ab
Control	0	0	0	1.13±0.35 bc	0.81±0.22 ab

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

Figure 6. Plant after *ex vitro* transfer (author)

6 Discussion

6.1 *In vitro* propagation

The most effective cytokinin for *in vitro* propagation of *Tacca integrifolia* was BAP. However, in *Tacca leontopetaloides*, zeatin was reported to be the most effective cytokinin (Cepková *et al*., 2015). Plants on medium without any cytokinin produced only 0.13 new shoots, which is similar to experiment of Charoensub *et al*., (2008), where there were not any new shoots in species *Tacca chantrieri.* However in the experiment of *Tacca integrifolia* the treatments with cytokinins BAP and zeatin increased number of shoot. The highest number of shoots was obtained on MS medium supplemented with 1.5 $mg.l^{-1}$ BAP (2.89) which corresponds to study of Charoensub *et al*., (2008), where *Tacca chantrieri* had the highest number of shoots (2.3) on medium supplemented with 2 $mg.l^{-1}$ BAP. According to Charoensub *et al*. (2008), plantlets of *Tacca chantrieri* produced callus and had abnormal shoot and less expanded leaves on MS (Murashige and Skoog, 1962) medium supplemented with concentrations 2 and 3 $mg.l^{-1}$ BAP, but plantlets of *Tacca integrifolia* started to form callus already at concentration 0.7 $mg.l^{-1}$ BAP. According to Martin *et al*., (2012) shoots of *Tacca leontopetaloides* tended to be hyper-hydric and form callus at the base of shoots with concentrations of BAP. *Tacca integrifolia* was not hyper-hydric and callus started to form from concentration 0.7 $mg.l^{-1}$ BAP. It can be assumed that increasing concentration of cytokinin decreases shoot elongation and tends to induce callus (Charoensub *et al*., 2008). According to Leshem *et al*. (1988), although cytokinin is needed to induce shoot proliferation, the supra-optimum concentrations can be toxic.

In *Dioscorea rotundata* the height of plants was evaluated after cultivation of plants on medium supplemented with BAP. The highest plant of *Dioscorea rotundata* were observed on control medium without plant growth regulator, the height of plantlets was decreased with increasing of BAP concentration (Ezeibekwe *et al*., 2009). This observation is different from experiment in *Tacca integrifolia*, where the highest plants were on medium with 1.5 $mg.l^{-1}$ BAP. According to study of Chen *et al.* (2007), the number of shoots was increased with increasing concentrations of BAP in

Dioscorea nipponica, which is completely in agreement with experiment of *Tacca integrifolia.*

Zeatin was the second cytokinin, which was used individually to induce production of adventitious shoots. In *Tacca leontopetaloides*, low concentrations of zeatin (0.1 and 0.3 $mg.l^{-1}$) produced normal shoots without any morphological abnormalities, but at concentrations 0.5 $mg.l^{-1}$ and higher, shoot formation decreased and started formation of callus (Cepková *et al*., 2015) while *Tacca integrifolia* accepted higher concentrations of zeatin without any morphological abnormalities; callus was formed from concentrations 0.7 $mg.l^{-1}$.

According to Ahanhanzo *et al*. (2010), yam (*Dioscorea spp.)* plants were lower on medium with high concentrations of zeatin. On the contrary, *Tacca integrifolia* plants were the highest from all experiment when cultivated on zeatin.

6.2 *In vitro* rooting and *ex vitro* transfer

On media with cytokinins, some roots were observed, although only few. These observations are not in agreement with experiment of Charoensub *et al*. (2008), where roots in *T. chantrieri* did not produce at all on medium with BAP and kinetin. In *T. integrifolia*, the highest number of roots was obtained on the medium where auxin (NAA) at concentrations 0.1 and 0.3 $mg.l^{-1}$ was used. According to Charoensub *et al*., (2008) medium with concentration 0.3 NAA $mg.l^{-1}$ was the most effective to increase formation of roots and this results were confirmed also in *Tacca integrifolia*. However, this concentration is higher than *Tacca leontopetaloides* needs for rooting. When higher concentrations of NAA in *Tacca leontopetaloides* were used, it led to decreased production of roots (Cepková *et al*., 2015). Nevertheless, maximum length of root was obtained on MS supplemented with 0.1 NAA $mg.l^{-1}$ in *Tacca integrifolia*, and on MS medium without auxin in *Tacca chantrieri* (Charoensub *et al*., 2008).

Also in some *Dioscorea* species, e.g., *D. remotiflora* and *D. zingiberensis*, a high concentration of NAA led to production of callus (Chen *et al*., 2003; Bernabe-Antonio *et al*., 2012) instead of rooting. According to Behera *et al*. (2008), in case of *Dioscorea hispida* there were not any roots on medium without auxins, and on medium supplemented with a concentration 0.5 $mg.l^{-1}$ NAA, there were only few roots. These

results are not congruent with experiment in *Tacca integrifolia*, where plants well-rooted already on medium supplemented with very low concentration of NAA (i.e., 0.1 $mg.l^{-1}$).

7 Conclusion

Regarding the fact that medium without PGRs did not produce new shoots, application of cytokinins seems crucial for *in vitro* propagation of *T. integrifolia*. The most appropriate medium for production of adventitious shoots was medium supplemented with 1.5 $mg.l^{-1}$ BAP providing approx. 2.89 of new shoots per explant.

For *in vitro* rooting, optimal results were obtained using medium supplemented with NAA. The most suitable concentration for effective rooting was 0.3 $mg.l^{-1}$ NAA. The average of number of new roots was 3.40±0.56 per explant and the percentage of rooting was 100%.

Ex vitro transfer of plants was successful when well-rooted plants, that had minimal two roots with minimally 1cm long, were transplanted garden substrate mixed with perlite in ratio 1:1. The percentage of survival plants after transferring to the *ex vitro* was 100%.

8 Recommendation

In this thesis, two types of cytokinins (BAP and zeatin) and one type of auxin (NAA) were tested. Within further research, effect of more types of PGRs (either individually of in combinations) might be tested on production of adventitious shoots and rooting. Two-step regeneration based on induction of organogenic callus and subsequent regeneration of plants from callus might be also very effective method for mass production of plants and optimization of this technique might be an objective of further study.

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