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In vitro propagation of *Disanthus cercidifolius* and assessment of genetic fidelity of regenerants

Master's thesis

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Declaration

27th April 2017

I hereby declare that this thesis entitled "*In vitro* propagation of *Disanthus cercidifolius* and assessment of genetic fidelity of regenerants" is my own work and all the sources have been quoted and acknowledged by means of complete references.

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Bc. Tereza Ulvrová

Acknowledgement

Abstract

In this study, an efficient micropropagation protocol has been developed for *Disanthus cercidifolius* Maxim. (Hamamelidaceae), a shrub originating in China and Japan, with attractive purple fall colour of heart-shaped leaves and great horticultural potential as ornamental shrub in parks and gardens. For this study, sprouting buds of two mature shrubs (genotype Truba and genotype PdS) were used as an initial plant material. For the *in vitro* propagation experiment, the nodal segments were cultured on a MS medium (Murashige and Skoog 1962) supplemented with either 6-benzylaminopurine (BAP) (0.5–3 mg.l^{-1}) or zeatin (0.5–3 mg.l^{-1}). As control, MS medium without plant growth regulators was used. The highest number of shoots per explant (in average 6.95 ± 0.33 in genotype PdS and 7.93 ± 0.41 in genotype Truba, respectively) was achieved on a medium supplemented with 2 mg.l^{-1} BAP. Two types of media, half-strength MS medium and half-strength WPM medium (Lloyd and McCown, 1980) supplemented with indole-3-butyric acid (IBA) (0.1–0.5 mg.l^{-1}), were tested for rooting of the shoots resulting in the best rooting performance on a half-strength WPM medium containing 0.5 mg.l^{-1} IBA. The rooted plantlets were transferred *ex vitro*, with 60% survival rate in genotype PdS and 33.3% in genotype Truba. Inter simple sequence repeat (ISSR) and flow cytometry were used in three shoots per 6 randomly chosen regenerants and two control plants (one for each genotype) to assess their genetic fidelity. Ten ISSR primers were used for molecular analysis and all amplified products were monomorfic indicating no genetic variability. Similarly, flow cytometric analysis showed that the ploidy level in all *in vitro* regenerants was stable and identical to mother plants. This analysis confirmed that micropropagation protocol optimized here represents a reliable method for the production of true-to-type plants. Therefore, after further improvement of *ex vitro* transfer, the protocol described here could be utilized for effective mass propagation of *Disanthus cercidifolius*.

Abstrakt

V rámci této práce byl optimalizován protokol mikropropagace dvoukvětce zmarličníkolistého [*Disanthus cercidifolius* Maxim. (Hamamelidaceae)], keře pocházejícího z Číny a Japonska, který vyniká svým podzimním okrasným zbarvením srdcovitých listů. Jako výchozí rostlinný materiál byly použity rašící pupeny ze dvou jedinců (genotyp PdS a genotyp Truba). Po povrchové sterilizaci pupenů byla založena sterilní *in vitro* kultura a rostliny byly pro účely pokusu pomnoženy průběžným pasážováním. K založení pokusu na *in vitro* množení byly využity jednonodální segmenty pěstované na MS médiu (Murashige and Skoog 1962) obsahujícím 6- benzylaminopurin (BAP) (0,5–3 mg.l^{-1}) nebo zeatin (0,5–3 mg.l^{-1}). Kontrolní variantou bylo MS médium bez přídavku růstových regulátorů. Nejvyššího počtu nových výhonů na rostlinu (v průměru 6,95 ± 0,33 u genotypu PdS a 7,93 ± 0,41 u genotypu Truba) bylo dosaženo při použitím média s přídavkem 2 mg.l^{-1} BAP. Pro zakořeňování výhonů byly testovány dva typy médií, poloviční MS médium a poloviční WPM médium (Lloyd and McCown, 1980), které obsahovaly auxin indol-3-máselnou kyselinu (IBA) (0,1–0,5 mg.l^{-1}). Nejlepší výsledky byly získány na polovičním WPM médiu s 0,5 mg.l^{-1} IBA. Následné převedení zakořeněných rostlin do *ex vitro* podmínek proběhlo s 60% mírou přežití u genotypu PdS a s 33,3% mírou přežití u genotypu Truba. Ověření genetické stability bylo testováno metodou Inter simple sequence repeat (ISSR) a stabilita ploidie průtokovou cytometrií. Obě analýzy probíhaly na třech výhonech ze šesti náhodně vybraných rostlin a dvě mateřské rostliny byly využity jako kontrola. ISSR analýza byla realizována s pomocí deseti primerů a všechny amplifikované produkty vyšly jako monomorfní. Průtoková cytometrie prokázala stejnou úroveň ploidie u všech *in vitro* regenerantů, shodnou s původním rostlinným materiálem. Na základě výsledků této studie lze shrnout, že tento protokol mikropropagace je vhodný pro množení druhu *D. cercidifolius* při zachování genetické stability získaných jedinců. Po optimalizaci *ex vitro* převodu lze tento postup využít pro efektivní množení tohoto keře.

Klíčová slova: *Disanthus cercidifolius*, genetická stabilita, *in vitro* množení, molekulární markery, růstové regulátory rostlin

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List of Abbreviations

AFLP   Amplified fragment length polymorphism

BAP   6-benzylaminopurine

CTAB   Cetyltrimethylammonium bromide

CULS   Czech University of Life Sciences

DAPI   4′,6-diamidino-2-phenylindole

DNA   Deoxyribonucleic acid

FAA   Formalin-acetic acid-alcohol

GA$_3$ Gibberellic acid

IAA   Indole-3-acetic acid

IBA   Indole-3-butyric acid

ISSR   Inter-simple sequence repeat

ITS   Internal transcribed spacer

MS   Murashige and Skoog medium (1962)

NAA   α-naphthaleneacetic acid

PCR   Polymerase chain reaction

PGR   Plant growth regulator

PVPP   Polyvinylpolypyrrolidone

RAPD   Random amplified polymorphic DNA

RFLP   Restriction fragment length polymorphism

SNP   Single nucleotide polymorphism

SRAP   Sequence-related amplified polymorphism

USA   Unites States of America

WPM   Woody plant medium (Lloyd and McCown, 1980)

ZEA   Zeatin

1   Introduction

Disanthus cercidifolius Maxim. from the Hamamelidaceae family is a deciduous shrub native to China and Japan. The shrub is cultivated mainly for ornamental purposes especially for its decorative fall colour of heart-shaped leaves (Missouri Botanical Garden, 2016; The Royal Horticultural Society, 2016). It has great potential to be used commercially in a larger scale as an ornamental shrub in parks and gardens, but optimization of mass propagation of plant material is needed.

D. cercidifolius can be propagated both sexual and asexual way. Unfortunately, both methods are very lengthy (Walter, 2001). Moreover, this species displays the reproductive phenomenon of "mass flowering but few fruiting" because of the limited types and quantities of pollination insects and low pollination efficiency (Xiao et al., 2009). Thus, *in vitro* propagation provides rapid and reliable system for production of large number of genetically uniform as well as disease-free plants and thereby it contributes to commercial plant propagation (Jha and Ghosh, 2005). In *Disanthus* genus, process of *in vitro* propagation has not been optimized yet.

Despite the advantages of the plant tissue cultures, genetic instability has been observed in several species after *in vitro* propagation (Das et al., 2010). Genetic variability is undesirable within the process of micropropagation. Thus, assessment of genetic fidelity of regenerants is essential for large scale multiplication and molecular markers represent efficient tool for detection of somaclonal variation. Flow cytometry allows the detection of ploidy level stability.

Therefore, the objective of this study was to develop an appropriate method of micropropagation, which could allow the regeneration of plants of *Disanthus cercidifolius* via direct morphogenesis and evaluation of genetic stability of *in vitro* regenerants using molecular markers and flow cytometry.

2 Literature review

2.1 Hamamelidaceae

Creation of the name is dated to 1818 when botanist Brown recognized it as a natural group (Li, 1997). Hamamelidaceae, also known as the Witch Hazel family, contains approximately 30 genera and about 140 species distributed in the tropical, subtropical and temperate areas in both the Old and New Worlds (Shi et al., 1998; Li et al., 1999). Spatial distribution of the family is illustrated in Figure 1.

Family Hamamelidaceae includes trees but more frequently shrubs (Novák and Skalický, 2012). Common characteristics are stipulate leaves, 2-carpellate pistils and multicellular stigmatic papillae (Li et al., 1999). Flowers are bisexual or unisexual situated in spikes, clusters or pairs (Cullen, 2001). Fruit is a loculicidal and septicidal woody capsule (Stevens, 2012; Encyclopaedia Britannica, 2016).

Members of the Witch Hazel family have great economic importance worldwide. Many species are large trees whose wood can be used as a lumber for different kinds of construction and furniture making (Li, 1997). Himalayan species *Parrotiopsis jacquemontiana* is used for making baskets and bridges (Encyclopaedia Britannica, 2016). Many shrubby species, such as *Hamamelis* (Li, 1997) or *Disanthus* (Encyclopaedia Britannica, 2016), are widely cultivated as ornamentals. Moreover, some species, such as *Hamamelis virginiana,* have medicinal use. It yields the widely used astringent and soothing lotion for cuts and bruises (Li, 1997).
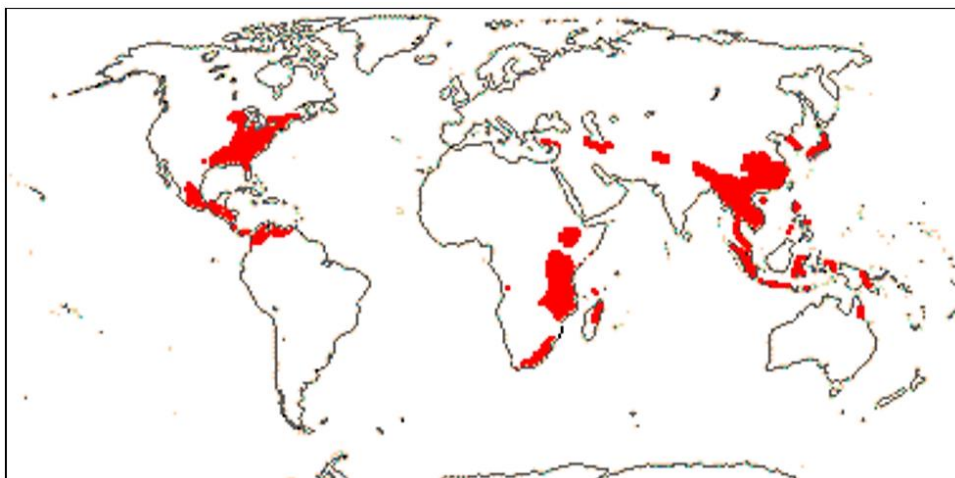
Figure 1: Distribution of Hamamelidaceae. Source: Stevens (2012)

2.2   *Disanthus cercidifolius*

2.2.1   Taxonomy

Disanthus Maxim. is a monotypic genus (Yu et al., 2014) containing single species *Disanthus cercidifolius* (Gao et al., 2009). The genus belongs to family Hamamelidaceae which is a member of order Saxifragales (Stevens, 2012). Families which are included in order Saxifragales are illustrated in Figure 2.

Figure 2: Division of order Saxifragales according to Stevens (2012)

According to Yu et al. (2014) genus *Disanthus* contains two different subspecies. First subspecies is *Disanthus cercidifolius* subsp. *longipes* occurring in China. Another subspecies is *Disanthus cercidifolius* subsp. *cercidifolius* which is endemic to Japan.

2.2.2   Nomenclature

Name of the genus comes from the Greek words *dis* meaning twice and *anthos* that means flower. Specific epithet is reference to the similarity of the leaves of *Disanthus* and *Cercis* (Missouri Botanical Garden, 2016).

Common names, which are used for *Disanthus cercidifolius*, are long-stiped disanthus (The Royal Horticultural Society, 2016) or just disanthus (Missouri Botanical Garden, 2016).

2.2.3   Origin and geographical distribution

Disanthus is a relict genus with a narrow area in Japan and China. It grows mainly in mountains up to 1000 m above sea level (Shatilova and Mchedlishvili, 2011). *Disanthus cercidifolius* subsp. *longipes* is distributed in a few regions in southern Hunan, central and north-western Jiangxi and southern Zhejiang Provinces of China (Xiao et al., 2007; Yu et al., 2014). It grows in mixed evergreen and deciduous broad-leaved forests in elevation from 450 up to 1200 m above sea level (Flora of China, 2003). Because of severe habitat fragmentation (Gao et al., 2009; Yu et al., 2014) it exists in a small number of individuals and it is ranked as a 2nd Class endangered species for conservation in China (Xiao et al., 2007). On the other hand, *Disanthus cercidifolius* subsp. *cercidifolius* is endemic to Japan (Flora of China, 2003; Yu et al., 2014).

2.2.4   Ecology

Disanthus thrives best in partial shade, but it tolerates both full shade (Missouri Botanical Garden, 2016) and full sun (The Royal Horticultural Society, 2016). The shrub prefers locations with protection from strong wind (Brickell, 2008; Missouri Botanical Garden, 2016). *Disanthus* is frost-resistant, but the foliage and new shoots may be damaged by late frosts. Suitable soils for right growth are acidic or neutral humus-rich and lime-free soils (Brickell, 2008; The Royal Horticultural Society, 2016). This genus is intolerant of drought (Missouri Botanical Garden, 2016) therefore soils should be moist but well-

drained (The Royal Horticultural Society, 2016). *D. cercidifolius* subsp*. longipes* prefers humid, acid soils and shady habitats (Yu et al., 2014).

2.2.5   Morphology

2.2.5.1 Habit

As shown in Figure 3, *D. cercidifolius* is a medium-sized, slender-branched, deciduous shrub which gradually spreads with age (Missouri Botanical Garden, 2016; The Royal Horticultural Society, 2016). The shrub usually has up to 3 m both in height and width (Brickell, 2008), but it can occasionally reach heights of 6 up to 8 m in forests when growing along streams (Yu et al., 2014).

Figure 3: Habitus of *Disanthus cercidifolius* cultivated in Arboretum Kostelec nad Černými lesy, Czech Republic. Source: Pokorný (2013)

2.2.5.2 Foliage

Leaves are alternate (Cullen, 2001) and long petiolate with large, linear, caducous stipules (Flora of China, 2003). Rounded, heart-shaped leaves (The Royal Horticultural Society, 2016) are thinly leathery or membranous and they are palmately 5-7 veined. Margins are entire (Flora of China, 2003).

As shown in Figure 4, blue green foliage changes colours in fall (Missouri Botanical Garden, 2016). Colours range from orange to purple to deep crimson (The Royal Horticultural Society, 2016) and the shrub can contain all shades at once (Brickell, 2008).

Figure 4: Fall foliage colour of *Disanthus cercidifolius*. Source: Coceano (2012)

2.2.5.3 Inflorescence

Genus *Disanthus* usually has inflorescences with 2 opposite flowers located axillary on short lateral branches (Figure 5) (Flora of China, 2003). The peduncles of inflorescence are approximately 8 up to 25 mm long. Flowers are bisexual (Xiao et al., 2009), slightly aromatic, tiny and dark purple in colour (Missouri Botanical Garden, 2016). Floral cup is short and abaxially brown hairy (Xiao et al., 2009). The flower contains 5 broader than long sepals, 5 red petals and 5 stamens (Flora of China, 2003) which are much shorter than petals (Xiao et al., 2009). The ovary is superior and produces 5 or 6 ovules per locule (Flora of China, 2003). The flowering period of *Disanthus* is from September to November (Xiao et al., 2009).

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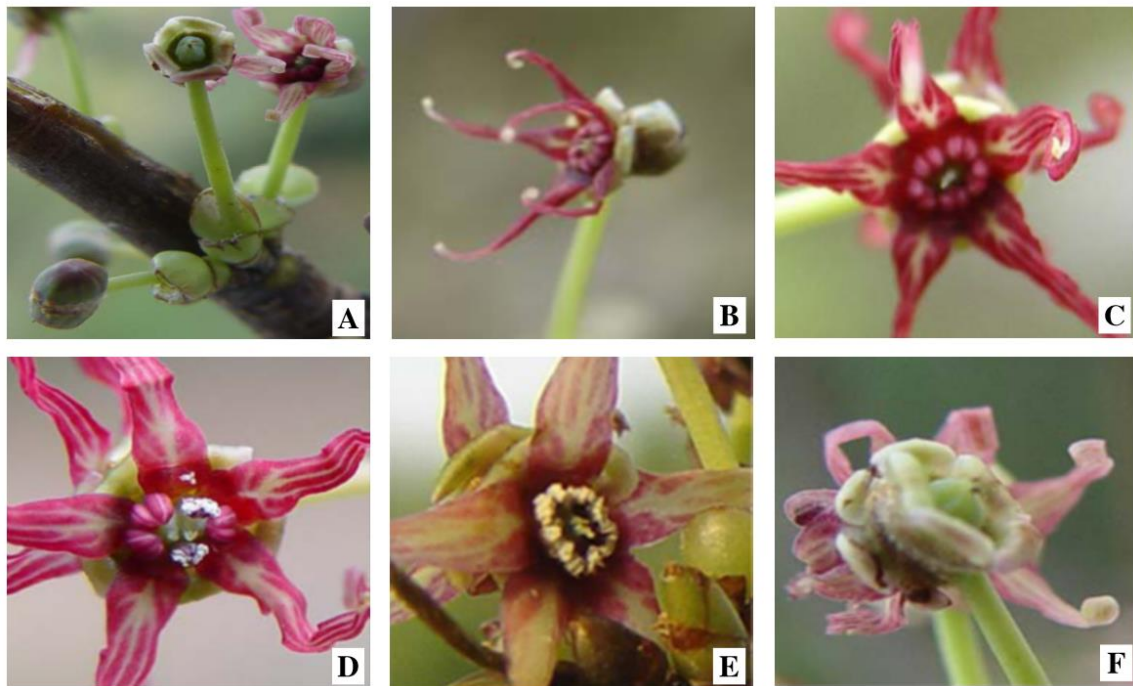
Figure 5: The floral morphological characteristics of *D. cercidifolius* subsp. *longipes* observed by Xiao et al. (2009). A – abnormality, three inflorescences growing paratactically at the axillae (*D. c.* subsp. *longipes* usually has two inflorescences which grow in opposite directions in the axillae at the same node); B – the two flowers on the same inflorescence opening differently; C – the two flowers on the same inflorescence are opening at the same time; D – two anthers are dehiscing; E – "full dehiscence", showing the "pollen ring"; F – "Withering period"

2.2.5.4 Fruits and seeds

Fruits of *Disanthus* are woody capsules dehiscing loculicidally by 2 valves. The endocarp of the fruit is bony and it is separated from the exocarp (Flora of China, 2003). Capsules become ripe in autumn of the year after flowering (Missouri Botanical Garden, 2016). Seeds are ellipsoid and unequal (Flora of China, 2003).

2.2.6 Reproductive biology of *Disanthus cercidifolius*

Pollination biology and seed production of *Disanthus cercidifolius* were studied on *D. c.* subsp. *longipes*.

Disanthus has a self-pollination and out-cross compatible breeding system and it has no agamospermy. It can be deduced that *Disanthus* had out-cross breeding system first and the self-cross and out-cross compatible breeding system evolved due to environmental

pressures. Inflorescence of this shrub has two flowers which usually blossom at the same time and blooming takes 6 up to 7 days. Most flowers bloom between the 15th up to 35th day after the first blossoming flower in the population (Xiao et al., 2009). According to Xiao et al. (2009) the typical flowering process can be divided into 4 periods: "Pre-dehiscence", "Initial dehiscence", "Full dehiscence" and "Withering". The calyces craze and two filaments lengthen during "Pre-dehiscence". The petal stretches completely and two filaments of the five are as tall as the styles during "Initial dehiscence" period. Moreover, one up to two anthers start to release pollen grains. "Full dehiscence" is characterized by dehiscing anthers and changes of the colour of the stigma to yellow. In addition to this, the other stamens release pollen grains which form a visible "pollen circle". This period occurs the third and fifth day of the flowering. "Withering" is last period, when all anthers are dehisced, the stamens are withered and the colour of some stigmas is changed to brown or black yellow. The nectar is presented at the base of the petals on the onset day or the second day of the flowering (Xiao et al., 2009).

Insect pollination is a more effective method of pollination than wind pollination in *Disanthus* genus. Wind pollination has very low efficiency and it just ensures reproductive success when insect pollination is not available (Xiao et al., 2009). According to Xiao et al. (2009) the main insect pollinators of *D. cercidifolius* subsp. *longipes* are *Episyrphus balteatus* (Diptera), *Scaptodrosophila coracina* (Diptera), *Polistes olivaceus* (Hymenoptera), *Apis cerana* (Hymenoptera), *Nezara viridula* (Hemiptera) and *Coccinella septempunctata* (Coleoptera) (classification according to Bisby et al., 2011). *Episyrphus balteatus* is an effective pollinator, because both visiting frequency and pollination efficiency of this species are high. Another effective pollinator is *Scaptodrosophila coracina*. Though this species has low pollination efficiency, the visiting frequency is high. Moreover, this species is presented in large numbers in place of natural distribution of *D. cercidifolius* subsp. *longipes* (Xiao et al., 2009).

Genus *Disanthus* displays the reproductive phenomenon of "mass flowering but few fruiting". This is a result of the limited types and quantities of pollination insects as well as low pollination efficiency. Effective pollinators shortage is probably an important reason why *Disanthus* is endangered (Xiao et al., 2009). According to Xiao et al. (2007), individuals with self-pollination have the lowest fruit and seed set, while cross-pollination has significantly higher fruit and seed sets. Xiao et al. (2007) also define that the

production of one fruit needs approximately 54.8 flowers, whereas the production of one seed needs 6.6 flowers or 83.9 ovules. Addition of fertilizer leads to lower abortive rate of the flower bud as well as it leads to a significantly higher fruit and seed set. Cutting the infirm and sick branches also contribute to higher fruit and seed set, even though the total number of blooming flowers is decreasing (Xiao et al., 2007).

2.2.7 Uses and properties

Disanthus cercidifolius is mostly cultivated as ornamental plant (Brickell, 2008). Missouri Botanical Garden (2016) describes *Disanthus* as "*an interesting and somewhat unusual shrub that is most often grown for its excellent fall colour*". However, the beautiful colour of the leaves is not sole ornamental aspect. Spidery and slightly smelly purple flowers grow on shrubs after leaf fall (Fischer, 2000; Brickell, 2008). They cannot compete with the foliage, but they are attractive in a quiet way (Fischer, 2000).

The shrub is commonly planted in the garden grove (Brickell, 2008) or in the shrub border as well as it is grown as a foundation plant or as a specimen around the home (Missouri Botanical Garden, 2016). Various examples of garden use of *Disanthus* are shown in Figure 6.

Figure 6: Garden uses of *D. cercidifolius*. Source: A, D – Bassuk and Trowbridge (2016); B – Sharp (2011); C – Hudson and Foley (2016)

2.2.8 Plant husbandry

2.2.8.1 Cultivation

The shrub should be planted in slightly acidic (Fischer, 2000) humus-rich, lime-free soil. Sand, clay and loam can be used as a substrate (The Royal Horticultural Society, 2016). As shown in Figure 7, medium moisture soils are suitable for cultivation of *Disanthus* (Missouri Botanical Garden, 2016). It is very important to ensure protection from harsh sun (Fischer, 2000) as well as protection from strong winds (Missouri Botanical Garden, 2016; The Royal Horticultural Society, 2016). Furthermore, when the shrub is planted in a sheltered position it is fully frost-hardy (Walter, 2001). Although it takes 10 up to 20 years to ultimate height (The Royal Horticultural Society, 2016), *Disanthus* needs enough space for cultivation, because it usually has up to 3 m both in height and width (Brickell, 2008).

Disanthus requires only minimal pruning. Just deviated or crossed branches should be cut off to maintain the shape of the crown. The ideal time to prune the shrub is late winter or early spring (Brickell, 2008).
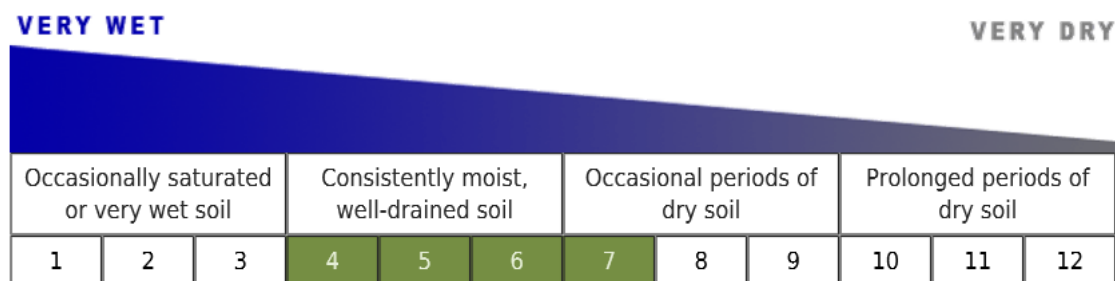
Figure 7: Moisture tolerance of *Disanthus cercidifolius*. Source: Bassuk and Trowbridge (2016)

2.2.8.2 Propagation

Disanthus can be propagated both sexual and asexual way. The first way is a sexual reproduction by seeds. The seed should be stratified immediately after harvest and sown in spring. Next year, seedlings should be transplanted to the seedling pots (Walter, 2001). According to Brickell (2008) and The Royal Horticultural Society (2016) seeds can be sown in an outdoor seedbed in spring or autumn. The whole process of sexual reproduction is very lengthy (Walter, 2001).

Vegetative propagation can be done by layering in spring (Brickell, 2008; The Royal Horticultural Society, 2016). But this process is lengthy as well as propagation by seeds. For this reason, grafting on root crown of seedlings of *Hamamelis virginiana* can be used (Walter, 2001). Fischer (2000) recommends the use of semihardwood cuttings treated with rooting hormone.

The problems connecting with traditional propagation methods, which were described both in Reproductive biology chapter and in this chapter, can be eliminated by using *in vitro* techniques. These techniques represent an effective plant propagation method as well as they eliminate pathogens and product healthy plant material (Ostrolucká et al., 2007).

2.2.8.3 Fertilization

In general, majority of woody plants does not require addition of fertilizer, when it is cultivated in ordinary garden soil. It mainly applies to soil which is enriched with humus before or during planting. Regular mulching and occasional addition of compost lead to sufficient supply of nutrients (Hensel et al., 2012).

According to Xiao et al. (2007) an addition of the fertilizer has a significant effect on lowering of the number of abortive flower buds as well as it leads to higher fruit and seed set. The composition of the fertilizer applied by Xiao et al. (2007) was $NO:P_2O_5:K_2O$ in a ratio of 3:3:2. The fertilizer was applied in two doses: 1,000 g of the fertilizer were applied around the root zone of the shrub and 0.5% solution of the fertilizer was sprayed on the leaves when the shrub was flowering (2,000 g of solution).

2.2.8.4 Pest and diseases

Although, the shrub is generally considered not to be very susceptible to pests (Brickell, 2008; The Royal Horticultural Society, 2016), it is susceptible to fungal diseases in hot, humid climates (Missouri Botanical Garden, 2016).

Motohashi et al. (2008) described *Phyllosticta disanthi* as a new species on *Disanthus* plants. The genus *Phyllosticta* includes endophytic fungi which are known as the causal

fungi of leaf spot diseases of various plants. The fungi cause typical light grey to pale brown leaf spots on *Disanthus* plants (Motohashi et al., 2008).

2.3   Use of *in vitro* propagation

Plant tissue culture, also known as *in vitro* culture, began to develop in 1940 (Thorpe, 2007). It is aseptic culture of cells, tissues and organs of plants on defined solid or liquid media under controlled conditions (Thorpe, 2007; Ponmurugan and Kumar, 2011). *In vitro* technologies are utilized to manipulate with plant germplasm, including clonal multiplication, generation of new variants as well as the production of genetically modified plants (Lynch, 1999).

An important use of tissue culture is micropropagation, especially in economical way (Ponmurugan and Kumar, 2011). It is a general term describing propagation of selected germplasm using *in vitro* techniques (Lynch, 1999). This method serves for propagation of large numbers of uniform plants, faster production of plants that are slow to propagate *in vivo* as well as to preservation and transportation of germplasm of many species including horticultural, medicinal and woody plants (Dunwell, 2010; Ponmurugan and Kumar, 2011). Whole process of micropropagation includes 4, actually 5 stages. Stage zero is preparative phase and donor plant is selected and prepared during this stage. Afterwards, another four stages follow: establishment of explant in culture, multiplication, rooting and transfer to natural environment (Lynch, 1999; Ponmurugan and Kumar, 2011).

2.3.1 Micropropagation in Saxifragales

In *Disanthus cercidifolius*, process of *in vitro* propagation has not been optimized yet. Moreover, plants from family Hamamelidaceae are not commonly used for micropropagation. It means that members of order Saxifragales, especially members of most related families to Hamamelidaceae (Figure 2), can provide an important data for research focused on micropropagation of *D. cercidifolius*. Techniques used to multiply plants *in vitro* in selected species related to this shrub are shown in Table 1.

Table 1: *In vitro* propagation techniques used in selected species taxonomically close to *Disanthus cercidifolius*

Technique	*Liquidambar* (Altingiacaeae)	*Cercidiphyllum* (Cercidiphyllaceae)	*Paeonia* (Paeoniaceae)	*Corylopsis* (Hamamelidaceae)	*Parrotiopsis* (Hamamelidaceae)	*Distylium* (Hamamelidaceae)
Micropropagation from axillary buds	Ďurkovič and Lux, 2010 Bayraktar et al., 2015	Fu et al., 2012	Beruto et al., 2004 Wen et al., 2016	Moon et al., 2002	Hussain et al., 2012	-
Somatic embryogenesis	Vendrame et al., 2001	-	Kim et al., 2006	-	-	Li et al., 2014
Direct organogenesis from various tissue explants	Erdag and Emek, 2005 Xu et al., 2007	Miaomiao et al., 2006	-	-	-	-
Indirect organogenesis	-	Chen et al., 2012	-	-	-	-

First step of micropropagation is establishment of *in vitro* culture, which means introduction of the surface disinfected explants into culture (Iliev et al., 2010). Erdag and Emek (2005) used lateral buds of 25-30 years old trees of *Liquidambar* placed on Woody plant medium (Lloyd and McCown, 1980) supplemented with 0.7 mg.l^{-1} BAP for establishment of *in vitro* culture. According to Beruto et al. (2004) and Wen et al. (2016) ideal medium for establishment of *in vitro* culture of tree peony (*Paeonia suffruticosa*) is WPM medium with addition of BAP as well. Axillary buds were used as initiating plant material in both researches. On the other hand, Ďurkovič and Lux (2010) established culture of *L. styraciflua* using sprouting axillary buds which were cultivated on WPM medium without plant growth regulators after surface sterilization. Bayraktar et al. (2015) compared the best medium for establishment of *in vitro* culture. They used 20 up to 30 cm long hardwood cuttings with axillary buds (0.5–0.7 cm) from suckers of *L. orientalis* which were kept in a 3 g.l^{-1} fungicide solution for 2 days. Afterwards, the cuttings were cut into pieces (each containing an axillary bud), surface sterilized, the primordial shoots were cut from the woody tissue and placed in glass tubes containing 10 ml of WPM medium or Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with various concentrations of different plant growth regulators. Based on their research, WPM medium containing 1 mg.l^{-1} BAP and 1 mg.l^{-1} IBA provided the best results for both shoot regeneration and shoot proliferation. Fu et al. (2012) achieved similar results during *in vitro* propagation of *Cercidiphyllum japonicum*. The percentage of shoot initiation was greatest when the explants (nodal sections of young shoots from the donor plant) were cultured on WPM supplemented with 1 mg.l^{-1} BAP and 0.01 mg.l^{-1} IBA.

Second stage of micropropagation is multiplication which is characterized by shoot proliferation and multiple shoot production (Iliev et al., 2010). Multiplication is encouraged by cytokinin alone or with a smaller amount of auxin (Ponmurugan and Kumar, 2011). Ďurkovič and Lux (2010) and Bayraktar et al. (2015) achieved the best results using the WPM medium with combination of BAP and IBA for shoot proliferation of *Liquidambar* species. Moreover, Bayraktar et al. (2015) proved that explant type influences number of shoots per explant. Based on their results, shoot clusters (three shoots per cluster) are more productive than single shoots in terms of shoot propagation. On the other hand, Erdag and Emek (2005) as well as Fu et al. (2012) recommend to use WPM medium supplemented with NAA and BAP for *in vitro* propagation of

Liquidambar orientalis and *Cercidiphyllum japonicum*. Wen et al. (2016) tested the effect of GA_3 on shoot multiplication of tree peony. The best results were achieved on WPM medium (double strength Ca^{2+}) supplemented with 1 mg.l^{-1} BAP and 0.5 mg.l^{-1} GA_3.

Root formation follows multiplication stage and shoots can be pretreated by elongation before rooting. Presence of auxins is significant for the induction of adventitious roots and rooting may be achieved either *in vitro* or *ex vitro* (Iliev et al., 2010; Ponmurugan and Kumar, 2011). Erdag and Emek (2005) transferred shoot clumps with microshoots (0.5 up to 1 cm) to WPM medium supplemented with 0.7 mg.l^{-1} BAP for shoot elongation before rooting. Elongated shoots in 2 up to 3 cm in length were best cultured for rooting on WPM containing 2 mg.l^{-1} IBA. Beruto et al. (2004) and Bayraktar et al. (2015) achieved the best results using the auxin IBA for *in vitro* rooting of tree peony and *Liquidambar orientalis* as well. Wen et al. (2016) examined impact of different polyamines (putrescine, spermine and spermidine) on rooting of tree peony. The best results were obtained using half-strength MS medium supplemented with IBA and 1 as well as 5 mg.l^{-1} putrescine. On the other side, Ďurkovič and Lux (2010) and Fu et al. (2012) found the best rooting performance in *Liquidambar styraciflua* and *Cercidiphyllum japonicum* using NAA.

Final stage is acclimatization during which regenerated plants are transferred to soil under natural environmental conditions (Iliev et al., 2010; Ponmurugan and Kumar, 2011). Erdag and Emek (2005) washed plantlets with sterile distilled water and transplanted them in plastic pots containing autoclaved sand and perlite mixture which were covered with clear plastic bags. Plantlets were kept in culture room and watered with WPM salts. Bayraktar et al. (2015) used similar protocol for acclimatization, but they used peat and loam mixture in pots and watered plants with diluted (1:10) WPM salts supplemented with 0.5 mg.l^{-1} BAP. Wen et al. (2016) tested application of arbuscular mycorrhizal fungi *Glomus mosseae* to improve plantlet establishment. Based on their results, plantlets which were inoculated with *G. mosseae* showed a high survival rate and vigorous growth in comparison with uninoculated plants.

2.3.1.1 Micropropagation in Hamamelidaceae

Hussain et al. (2012) examined micropropagation of *Parrotiopsis jacquemontiana*, a small deciduous tree from family Hamamelidaceae. Both shoot apices and nodal segments were surface sterilized in 0.1% $HgCl_2$. According to them, the shoot formation percentage was highest on MS medium supplemented with 1.1 mg.l^{-1} BAP and shoot multiplication was best on MS medium supplemented with both 1.1 mg.l^{-1} BAP and 0.35 mg.l^{-1} IAA. Roots were best induced on half-strength MS medium with IBA.

Moon et al. (2002) developed an *in vitro* micropropagation system via shoot formation of *Corylopsis coreana*. Based on their results, the highest shoot proliferation was obtained on MS medium supplemented with 0.5 up to 3 mg.l^{-1} zeatin and 0.2 mg.l^{-1} BAP. Half-strength MS medium containing 0.5 mg.l^{-1} NAA was used for *in vitro* rooting. Moreover, research of Moon et al. (2002) showed that juvenile explants (one year old seedlings) are better in booth shoot proliferation and rooting than mature explants (ten years old tree).

2.3.1.2 Use of *in vitro* techniques in *Disanthus cercidifolius*

Just primary study on micropropagation focused on illumination spectrum was done in *Disanthus cercidifolius*. Marks and Simpson (1999) modified growth of *Disanthus cercidifolius* by altering either the spectral quality or the level of irradiance received by shoot cultures. Shoot tips were dissected aseptically from apical buds and grown on Linsmaier and Skoog medium (Linsmaier and Skoog, 1965) containing half-strength MS macro-elements supplemented with 0.7 mg.l^{-1} BAP. Their results showed that shoot elongation is supported by red light. On the other hand, shoot length was reduced significantly at irradiances of 55 and 106 µmol m^{-2}.s^{-1}. Moreover, severe shoot tip necrosis occurred at 106 µmol m^{-2}.s^{-1}, with less necrosis at 55 µmol m^{-2}.s^{-2} and none at 11 µmol m^{-2}.s^{-1} and 26 µmol m^{-2}.s^{-1}.

2.4 Somaclonal variation and evaluation of genetic stability of *in vitro* regenerants by molecular markers

Genetic instability has been observed in several species after *in vitro* propagation (Das et al., 2010). Genetic variability is undesirable in the process of micropropagation and it should be minimized as possible (Pavlová, 1992), because production of true-to-type plants is important to retain and certify the clonal fidelity for mass multiplication (Naing et al., 2013). Therefore, the maintenance of genetic stability of *in vitro* regenerants is an essential requisite for large scale production (Das et al., 2010).

Karp (1995) summarized the main factors influencing somaclonal variation. First factor is the degree of departure from organised meristematic growth. According to Karp (1995) the greater the departure from organised growth and the longer the time spent in this state, the greater the chances of generating somaclonal variation. Second factor is the genetic constitution of the starting material, because somaclonal variation is genotype-dependent and furthermore, more somaclonal variation, at least in terms of chromosome instability, is recovered in regenerants of polyploids compared with diploids and haploids. The choice and particularly the concentration of growth regulators in the medium influence variation and they are the third factor. Finally, somaclonal variation is dependent on tissue source. The older or the more specialised the tissue, the greater the chances of variation will be recovered in the regenerated plants. In addition to this, according to Krishna et al. (2016) the longer a culture maintained *in vitro*, the greater the somaclonal variation.

A wide range of tools is available for the detection and characterization of somaclonal variants which are based on the differences in morphological traits, cytogenetical analysis, biochemical and molecular DNA markers or their combinations (Harding, 1999; Krishna et al., 2016). Advantages and disadvantages of different marker systems for the assessment of clonal fidelity are described in Table 2.

Table 2: Strengths and weaknesses of different marker systems for the assessment of clonal fidelity. Source: Krishna (2016)

Advantages	Disadvantages
Morphological traits	
Visual differentiation	Sensitive to ontogenic changes and other environmental
Does not require any laboratory facility	factors
Suitable for preliminary detection	Limited in numbers
	Time-consuming
Cytological markers (flow-cytometry)	
Sample preparation and analysis is convenient and rapid in case of in flow-cytometry	Cytosolic compounds may interfere with quantitative DNA staining in flow-cytometry
Rapid and efficient method for routine large-scale studies of ploidy level	Absence of a set of internationally agreed DNA reference standards in case of in flow-cytometry
Unfailing detection of even the smallest modifications in chromosome number	Time-consuming chromosome counting
Isozyme markers	
Codominant expression	Sensitive to ontogenic changes and other environmental
Ease of performance	factors
	Limited in numbers
	Not all of these reagent systems work efficiently with all plant species
	Tissue-specific expression
DNA markers	
Codominant expression	RAPD markers are dominant and do not permit the scoring of heterozygous individuals. Besides, they exclusively identify sequence changes
Any source DNA can be used for the analysis	
Phenotypically neutral	Possible non-homology of similar sized fragments as ISSR is a multilocus technique
Not sensitive to ontogenic changes and other environmental factors	Disadvantages of AFLPs include the need for purified, high molecular weight DNA, the dominance of alleles and the possible non-homology of comigrating fragments belonging to different loci
Capability to detect culture-induced variation both at the DNA sequence and methylation pattern levels	Involvement of high development costs in SSR markers if adequate primer sequences for the crop species of interest are unavailable. Further, mutations in the primer annealing sites may result in the occurrence of null alleles, which may lead to errors in scoring

Molecular markers are tissue and environmental independent. Therefore, DNA-based markers are more reliable than morphological and isozymic markers (Sreedhar et al., 2007). Various molecular approaches such as AFLP (amplified fragment length polymorphism), RAPD (random amplified polymorphic DNA), RFLP (restriction fragment length polymorphism) have been attempted to identify and measure the level of somaclonal variation in *in vitro* regenerants (Sharma et al., 2007). According to Sreedhar et al. (2007) RFLP involves high cost, radioactivity and it is laborious. Although, Sharma (2007) stated that AFLP is one of the more robust molecular techniques for cultivar identification and variability analysis and it offers the best chance for detecting tissue culture-induced changes, according to Sreedhar et al. (2007) it is not suitable for routine application for tissue cultured plants and it is costly. On the other hand, RAPD is simpler

and has proven to be quite efficient in detecting genetic variations. Sreedhar et al. (2007) also stated that ISSR (inter-simple sequence repeat) is a very simple, quick, cost-effective, highly discriminative and reliable method which combines both advantages of AFLP and the universality of RAPD.

One of the most commonly used method to access somaclonal variation is flow cytometry as well (Brito et al., 2010), which is used to study of ploidy level on a large scale (Naing et al., 2013). Flow cytometry allows easy as well as rapid analysis (Loureiro et al., 2005) and thus it has been used to access clonal fidelity in several woody species (Brito et al., 2010).

2.4.1 Assessment of genetic fidelity of regenerated plants in Hamamelidaceae

Li et al. (2014) assessed genetic fidelity of the micropropagated plants of *Distylium chinense* regenerated via somatic embryogenesis. Leaves of three parental plants, six regenerated plants, six embryogenic callus lines and six somatic embryo lines were used for analysis and genomic DNA was extracted by modified cetyltrimethylammonium bromide method (CTAB) (Doyle and Doyle, 1989). Genomic DNA was then PCR-amplified using SRAP (sequence-related amplified polymorphism) primers. According to their results, all regenerated plants showed 100% genetic similarity with the parental plant as well as with each other. Only minor variation with absence of certain parental bands was presented in embryogenic callus line. The study supports the use of SRAP-PCR which gives rapid indication of the level of genetic stability in plants regenerated *in vitro*.

Members of family Hamamelidaceae are not commonly used for micropropagation, therefore assessment of genetic fidelity of regenerated plants is not very explored. On the other hand, molecular markers are widely used for analysis of genetic diversity. Examples of application of molecular markers in family Hamamelidaceae are shown in Table 3.

Table 3: The molecular marker techniques used in Hamamelidaceae

Species	Molecular marker technique	Reference
Corylopsis coreana	RAPD, ITS	Roh et al., 2007
Disanthus cercidifolius	ISSR AFLP	Xie et al., 2010 Yu et al., 2014
Distylium chinense	ISSR SRAP	Li et al., 2011 Li et al., 2014
Fothergilla spp.	SNP	Qi et al., 2015
Loropetalum chinense	RAPD AFLP	Chendong et al., 2002 Gong et al., 2016
Loropetalum subcordatum	AFLP	Gong et al., 2010
Parrotia spp.	ITS	Li et al., 1997
Sinowilsonia henryi	ISSR AFLP	Zhou et al., 2014 Zhang et al., 2015

3 Aims of the thesis

The aim of the diploma thesis was to optimize the process of micropropagation of an ornamental shrub *Disanthus cercidifolius*, and detection of genetic fidelity of *in vitro* regenerants using molecular markers and flow cytometry.

In this context, the partial objectives of the thesis were following:

- Establishment of *in vitro* culture from initial plant material (*D. cercidifolius*).
- Development of appropriate protocol for induction of *in vitro* direct morphogenesis by testing various concentrations and types of cytokinins.
- Induction of *in vitro* rooting of shoots.
- *Ex vitro* transfer of plantlets.
- Evaluation of genetic fidelity of regenerated plants using molecular markers and flow cytometry.

The objectives of the diploma thesis were set under following hypothesis:

- Zeatin and 6-benzylaminopurine are cytokinins stimulating cell division and shoot proliferation and thus they will induce shoot development in *D. cercidifolius*.
- Indole-3-butyric acid will induce rooting in *D. cerdicifolius*.
- Micropropagation via direct morphogenesis minimalizes the possibility of occurrence of somaclonal variation, therefore *in vitro* regenerants of *D. cercidifolius* will be genetically uniform.

4   Material and methods

4.1   Plant material

Sprouting buds from mature shrubs of *Disanthus cercidifolius* Maxim. were used as an initial plant material (Figure 8). Two genotypes of the shrub were used for the experiment. First genotype Truba was obtained from the Arboretum in Kostelec nad Černými lesy in the Czech Republic, where collected material was introduced in 1979 (seeds were received from Washington Park Arboretum in Seattle). Second genotype, named PdS, originates from the Arboretum Wespelaar and garden of Herkenrode in Belgium (the shrub was planted from cuttings in 1995).

Figure 8: Sprouting buds of *Disanthus cercidifolius* used for establishment of the culture. Source: author

4.2   Methods

4.2.1   Establishment of *in vitro* culture

The establishment of *in vitro* culture of *D. cercidifolius* was carried out in the Laboratory of Plant Tissue Cultures of the Department of Crop Sciences and Agroforestry at the Faculty of Tropical AgriSciences of the Czech University of Life Sciences Prague during April.

Sprouting buds of *D. cercidifolius* were collected from mature shrubs and sterilized in an aqueous solution of 0.5% $HgCl_2$ for 6 minutes. Afterwards, the plant material was three times rinsed with sterilized distilled water and transferred on the MS medium (Murashige and Skoog, 1962) supplemented with 30 g.l^{-1} sucrose, 8 g.l^{-1} agar, 100 mg.l^{-1} *myo*-inositol, 200 mg.l^{-1} casein, 200 mg.l^{-1} L-glutamine and 1 mg.l^{-1} BAP (6-benzylaminopurine). The pH of medium was adjusted to 5.7 with 1 M KOH before pouring into 100 ml Erlenmeyer flasks and autoclaving at 121 °C and 100 kPa pressure for 20 minutes.

Explants were maintained at 25/23 °C under a 16/8 h light/dark photoperiod provided by cool white fluorescent lamps (36 μmol m^{-2}.s^{-1}).

After bud germination, the plants were regularly sub-cultivated on the medium of the same composition and cultivated under the conditions described above. Explants were multiplied by both nodal and apical segments every 3-4 weeks until sufficient plant material was prepared for the experiments.

4.2.2 Testing of media for formation of adventitious shoots

Nodal segments approximately 0.5 cm in height (Figure 9) of two genotypes of *D. cercidifolius* containing one axillary bud were exposed to 8 different treatments with various concentrations of plant growth regulators (Table 4) and one control. The nodal segments were taken strictly from middle part of shoots in order to standardize the experiment and they were cultivated on MS medium supplemented with 100 mg.l^{-1} myo-inositol, 200 mg.l^{-1} casein, 200 mg.l^{-1} L-glutamine and different PGRs at various concentrations, solidified with 8 g.l^{-1} agar and 30 g.l^{-1} sucrose added as a carbohydrate source. Zeatin and BAP at concentrations from 0.5 mg.l^{-1} to 3 mg.l^{-1} were added in the medium. As control, MS medium without plant growth regulators was used.

Cultures were maintained under same conditions as described above. Height of the plant, number of offshoots, length of offshoots and number of buds per plant were measured after 12 weeks of the culture.

Table 4: Types and concentrations of cytokinins added in MS medium and used for *in vitro* propagation of *D. cercidifolius*

Treatment	Plant growth regulator (mg.l^{-1})	
	BAP	ZEA
1.	0.5	
2.	1.0	
3.	2.0	
4.	3.0	
5.		0.5
6.		1.0
7.		2.0
8.		3.0
Control	0	0
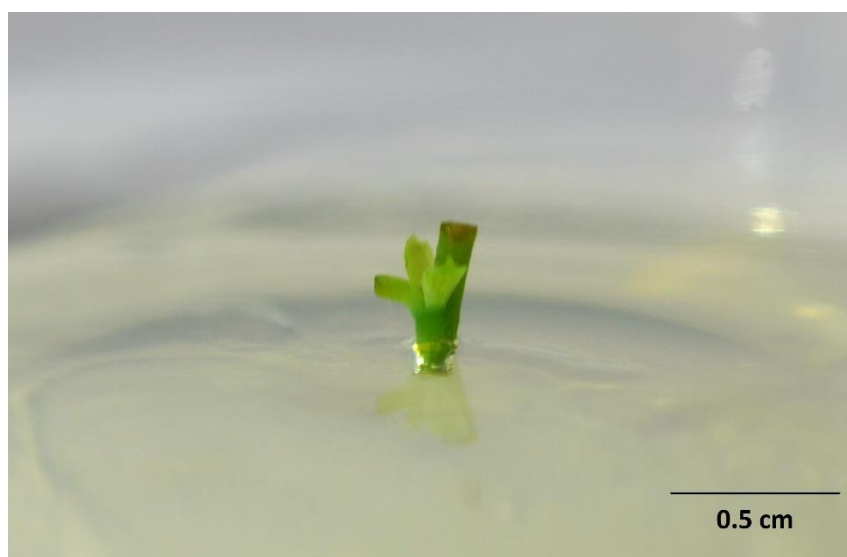
Figure 9: The nodal segment with single axillary bud of *Disanthus cercidifolius* used for *in vitro* propagation. Source: author

Experiments were set up with 20 repetitions of each genotype per treatment and repeated twice. The optimal treatment for *in vitro* propagation was selected, and to reveal potential somaclonal variation occurring in various shoots of the same plant, three shoots per three randomly chosen plants of each genotype were subjected ISSR and flow cytometric analyses.

4.2.2.1 Histological analysis

Since callus on the explant basis developed and it was not evident whether new microshoots regenerate from the primary explant or from callus, the morphological origin of the adventitious shoots was studied using histological analysis and microscopy. The analysis was carried out in the Laboratory of Biologically Active Compounds of the Institute of Experimental Botany of the Academy of Sciences of the Czech Republic in Prague during January and February 2017.

For histology, adventitious shoots with calli were randomly collected from regenerants developed on MS medium supplemented with 2 mg.l^{-1} BAP and they were cut into smaller pieces. The pieces were fixed in standard FAA fixative solution (50% of ethanol, 5% acetic acid, 5% formalin and 40% distilled water) for 24 hours. After fixation, samples were dehydrated in sequenced aqueous ethanol and butanol solutions (Table 5). The samples were left in each grade for a period of at least 2 hours.

Afterwards, paraffin was infiltrated into the tissues by applying paraffin gradually to the medium of pure 100% butanol containing dehydrated samples at room temperature, 40 °C and 58 °C. The whole process of paraffin infiltration took 9 days. Subsequently, paraffin blocks were prepared by embedding the specimens in molten paraffin wax employing the embedding centre Leica EG1150H (Leica, Germany).

The paraffin blocks were sectioned into 12 µm slices using a microtome (Leica, Germany). Each section was placed on the glass slide which was smeared with solution of glycerol and egg white and covered with distilled water. After evaporation of the water, samples were ready for staining.

Table 5: Steps of dehydration of samples of *D. cercidifolius* for histological analysis

Step	Distilled water	Ethanol	Butanol
1	45%	30%	25%
2	30%	30%	40%
3	20%	25%	55%
4	10%	20%	70%
5	-	15%	85%
6	-	-	100%

Two protocols were used for staining. Alcian blue, which stains mucopolysaccharides as cellulose cell walls and pectins, was used in both procedures. Nuclear fast red staining nuclei was used in first protocol and Safranin staining lignified tissues was used in second protocol. Individual steps of staining procedure are shown in Table 6.

The stained slides were mounted by coverslips using resin and they were observed under compound microscope Jenaval (Carl Zeiss Microscopy, USA) as well as under stereo microscope Nikon SMZ1500 (Nikon, Japan). The pictures were taken by camera Nikon Digital Sight DS-U1 (Nikon, Japan).

Table 6: Staining procedure for histological analysis of *D. cercidifolius*

First protocol		Second protocol	
Solution	Duration	Solution	Duration
Citrus clearing solvent I	3 min.	Citrus clearing solvent I	3 min.
Citrus clearing solvent II	3 min.	Citrus clearing solvent II	3 min.
Citrus clearing solvent III	3 min.	Citrus clearing solvent III	3 min.
Citrus clearing solvent / 100% Et-OH (1:1)	3 min.	Citrus clearing solvent / 100% Et-OH (1:1)	3 min.
100% Et-OH	3 min.	100% Et-OH	3 min.
100% Et-OH	3 min.	100% Et-OH	3 min.
96% Et-OH	3 min.	96% Et-OH	3 min.
70% Et-OH	3 min.	70% Et-OH	3 min.
50% Et-OH	3 min.	50% Et-OH	3 min.
30% Et-OH	3 min.	30% Et-OH	3 min.
Distilled water	3 min.	Distilled water	3 min.
3% acetic acid	Rinse	3% acetic acid	Rinse
0.1% Alcian Blue in 3% acetic acid	15 min.	0.1% Alcian Blue in 3% acetic acid	35 min.
Distilled water	5 min.	Distilled water	5 min.
Nuclear Fast Red	10 min.	30% Et-OH	Rinse
Distilled water	5 min.	1% Safranin in 50% Et-OH	20 min.
30% Et-OH	Rinse	-	-
50% Et-OH	1 min.	50% Et-OH	20 min.
70% Et-OH	2 min.	70% Et-OH	-
96% Et-OH	2 min.	96% Et-OH	-
100% Et-OH	2 min.	100% Et-OH	Rinse
100% Et-OH	2 min.	100% Et-OH	1 min.
Citrus clearing solvent / 100% Et-OH (1:1)	2 min.	Citrus clearing solvent / 100% Et-OH (1:1)	2 min.
Citrus clearing solvent III	2 min.	Citrus clearing solvent III	2 min.
Citrus clearing solvent II	2 min.	Citrus clearing solvent II	2 min.
Citrus clearing solvent I	2 min.	Citrus clearing solvent I	2 min.

4.2.3 Root induction and *ex vitro* transfer

Microshoots, more than 1.5 cm in length, were excised from shoot proliferating cultures and transferred to rooting medium. Half-strength MS medium and half-strength WPM medium with addition of different concentrations of IBA (indole-3-butyric acid), 15 g.l^{-1} sucrose and 8 g.l^{-1} agar were tested for induction of roots. As control, half-strength MS as well as WPM medium without plant growth regulators were used (Table 7). Cultures were maintained under same conditions as plants for shoot multiplication.

Table 7: Types of media and concentrations of auxin used for *in vitro* propagation of *D. cercidifolius*

Treatment	Medium	IBA (mg.l^{-1})
1.	½ MS	0.1
2.	½ MS	0.3
3.	½ MS	0.5
4.	½ WPM	0.1
5.	½ WPM	0.3
6.	½ WPM	0.5
Control	½ MS	0
Control	½ WPM	0

Each treatment consisted of 20 repetitions from every single genotype. Number of roots as well as root length were measured after 6 weeks of culture.

The *ex vitro* transfer was carried out in the Botanical Garden of the Faculty of Tropical AgriSciences of CULS Prague in 2017. Together, 45 well-rooted plants (at least two roots 0.5 cm in length), 15 plants from genotype Truba and 30 plants from genotype PdS, were transferred *ex vitro* after 6 weeks of cultivation on rooting medium. Plants were removed carefully from Erlenmeyer flasks, roots were rinsed under tap water and whole plats were transferred into flower pots containing a sterilized mixture of garden substrate:peat:perlite

(1:1:1). The flower pots with plants were placed in small glasshouses to maintain high air humidity and the plants were uncovered gradually after couple of days. Survival rate was evaluated after a month.

4.2.4 Statistical analysis

Statistical analysis of data obtained from micropropagation was performed by analysis of variance (ANOVA), and the significantly different means were identified by using Tukey's HSD test at the 5% level of significance ($P \leq 0.05$) (STATISTICA 12.0, StatSoft).

4.2.5 DNA extraction and ISSR analysis

Assessment of the genetic fidelity of regenerated plants was carried out in the Laboratory of Molecular Genetics of the Faculty of Environmental Sciences of CULS Prague in 2017.

Three shoots from three randomly selected plants from each genotype (i.e. 18 samples) cultivated on MS medium supplemented with 2 mg.l^{-1} BAP were chosen for extraction of DNA and subsequent ISSR analysis. The original plant material from each genotype was used as a control.

For ISSR analysis (Zietkiewicz et al. 1994), genomic DNA of leaf samples of *Disanthus cercidifolius* was extracted with CTAB method (Doyle and Doyle, 1989) and the protocol was modified to include a trace of polyvinylpolypyrrolidone (PVPP) and 5 µl of RNase of concentration 10 mg.µl^{-1} (Thermo Scientific, Czech Republic). DNA quality was determined by 1.5% agarose gel electrophoresis in 1× TBE buffer. DNA concentration was measured on a UVS-99/UVISDrop (Avans Biotech, Taiwan). The isolated DNA samples of *D. cercidifolius* were prepared in aliquots of the individual samples with concentration 50 ηg.µl^{-1} for PCR, and stored at -20°C.

A set of 10 ISSR primers (University of British Columbia, USA) was used for screening. DNA amplifications using the PCR were carried out in a reaction volume of 20 µl with the following composition: 10 µl × PPP Master Mix [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)], 7.3 μl PCR H$_2$O (Top-Bio, Czech Republic), 2 μl template DNA (50 ng.μl^{-1}), 0.5 μl primer (0.1 μM), and 0.2 μl bovine serum albumin (20 mg.ml^{-1}) (Thermo Scientific, USA). PCR reactions were performed in a Veriti 96 Well Thermal cycler (Applied Biosystems, USA) and annealing temperatures were modified to optimize the reaction for individual primers (Table 10). The cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing temperatures ranged from 49 to 54 °C for 1 min, and extension at 72 °C for 2 min, with the final extension at 72 °C for 8 min. Amplified products were electrophoretically separated on 1.5% agarose gel using Enduro power station (Labnet international, USA). Gels were stained with 2 μl ethidium bromide (Carl Roth GmbH, Germany) with concentration 0.5 μl.ml^{-1} and they were run at 100 V for approximately 1.5 hour. The size of the amplified products was estimated using a GeneRuler 100 pb Plus DNA Ladder (Thermoscientific, Lithuania) and the amplified stained products were visualized on a gel with a Syngene GENi2 UV transilluminator (Trigon plus, Czech Republic). ISSR profiles were scored visually as either having the presence (1) or absence (0) of bands in the gel. Only clear, distinct bands were scored.

4.2.6 Analysis of ploidy level

For the analysis, 18 randomly selected adventitious shoots obtained from 3 *in vitro* regenerants per genotype and two control plants (both genotypes) were used. As internal standard, *Bellis perennis* (2C = 3.38 pg; Schönswetter et al., 2007) was used. Ploidy levels were detected by flow cytometry using the modified two-step methodology according to Doležel et al. (2007).

In brief, to release nuclei from the cells, approximately 1 cm^2 of each sample of *Disanthus cercidifolius* was chopped with a sharp razor blade together with a young leaf of the internal reference standard in 1 ml of ice-cold Otto I buffer containing 0.1 M citric acid and 0.5% Tween 20 (Otto, 1990). The suspension of nuclei was then filtered through a 42 μm nylon mesh to remove fragments and large tissue debris. Subsequently, 1 ml of staining buffer Otto II containing 0.4 M Na$_2$HPO$_4$.12H$_2$O (Doležel and Göhde, 1995) and

supplemented with AT-selective fluorescent dye DAPI (4´,6-Diamidino-2-phenylindole) as well as 2-mercaptoethanol in final concentrations of 4 µg.ml^{-1} and 2 µl.ml^{-1} was added. After short incubation period at room temperature, at least 3,000 nuclei were analysed in a CyFlow Space flow cytometer (Partec GmbH, Münster, Germany). Data were evaluated using the FlowMax software (Partec, GmbH, Münster, Germany) and the DNA-ratios were counted as the ratio between the G0/G1 peaks of the studied plant and the internal standard.

5   Results

5.1   Establishment of *in vitro* culture and multiplication of plant material

The applied treatment for surface sterilization using 0.5% HgCl$_2$ provided 12 uncontaminated plantlets from 13 used sprouting buds (i.e. 92.3% efficiency). Plantlets on MS medium supplemented with 1 mg.l^{-1} BAP began to grow within two weeks and they were transferred to fresh medium after one month (Figure 10). Overall, during regular sub-cultures, plants regenerated more effieciently from nodal segments than from apical segments (data not shown). Plant material multiplied via this process, was used for the experiment.
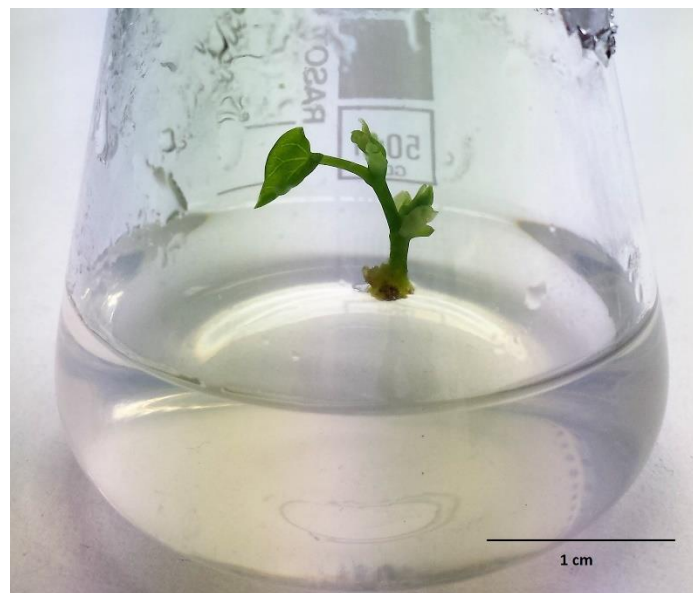
Figure 10: The plantlet one month after surface sterilization.
Source: author

5.2 *In vitro* multiplication of plants

Adventitious shoots started to develop from axillary buds of explants from the second up to third week of culture in most of the treatments. Thereafter, the shoots developed asynchronously depending on the treatment and resulting in formation of a cluster composed of shoots of various sizes.

After 12 weeks of cultivation, plants of both genotypes grown on a medium supplemented with 2 mg.l^{-1} BAP produced the highest number of new shoots per explant (in average 6.95 ± 0.33 in genotype PdS and 7.93 ± 0.41 in genotype Truba, respectively) with statistically significant differences in comparison to other treatments (Figure 11). Plants grown on this treatment were superior in number of buds per explant as well (in average 30.90 ± 1.23 in genotype PdS and 31.25 ± 1.52 in genotype Truba, respectively), which is an important indicator for *in vitro* propagation. The average plant height was somewhat lower at this concentration in comparison with some other treatments (i.e. 0.5 mg.l^{-1} BAP in genotype PdS, 2 mg.l^{-1} and 3 mg.l^{-1} ZEA in genotype Truba), but statistically significant differences were not detected among these treatments. Lower and higher concentrations of this BAP decreased shoot multiplication (Table 8). Moreover, higher concentrations of BAP resulted in hyper-hydration of shoots (Figure 12).

Although, zeatin provided faster initial growth (first shoots appeared a week earlier), BAP proved to be more effective for i*n vitro* propagation of *Disanthus cercidifolius* than did zeatin in total. Zeatin did not provide sufficient amount of shoots as well as buds per explant, and furthermore, plants did not grow but rather withered since the 8. week of cultivation (Table 8).

Most of the plants in all treatments except control produced callus surrounding the basal part of the the explant. Therefore histological analysis was needed to determine the morphological origin of the shoots.

Table 8: Effect of plant growth regulators on plant height, number of shoots, number of buds and length of shoots in two genotypes (PdS, Truba) of *Disanthus cercidifolius* after 12 weeks of culture

PGR (mg.l⁻¹)		Height of explant (cm) (mean ± SE)		Number of shoots per explant (mean ± SE)		Number of buds per explant (mean ± SE)		Length of shoots (cm) (mean ± SE)	
BAP	ZEA	PdS	Truba	PdS	Truba	PdS	Truba	PdS	Truba
0	0	0.70 ± 0.05 a	0.68 ± 0.03 b	0.60 ± 0.08 d	0.48 ± 0.08 f	1.08 ± 0.19 b	0.80 ± 0.15 b	0.39 ± 0.08 b	0.42 ± 0.05 a
0.5		2.57 ± 0.10 c	1.86 ± 0.07 de	2.83 ± 0.15 c	3.05 ± 0.22 cd	14.10 ± 0.67 d	12.18 ± 0.81 c	1.72 ± 0.05 e	1.18 ± 0.04 ef
1		2.44 ± 0.08 c	2.02 ± 0.05 cde	5.10 ± 0.32 b	5.65 ± 0.31 b	24.98 ± 1.34 c	22.28 ± 1.21 e	1.49 ± 0.03 cd	1.13 ± 0.03 f
2		2.44 ± 0.06 c	2.13 ± 0.06 cd	6.95 ± 0.33 a	7.93 ± 0.41 a	30.90 ± 1.23 a	31.25 ± 1.52 a	1.43 ± 0.03 c	1.21 ± 0.03 ef
3		1.99 ± 0.04 b	1.87 ± 0.07 de	5.63 ± 0.35 b	4.75 ± 0.36 b	24.08 ± 1.26 c	18.75 ± 1.39 e	1.28 ± 0.02 a	1.34 ± 0.03 de
	0.5	2.34 ± 0.10 bc	1.83 ± 0.06 e	1.10 ± 0.05 df	1.15 ± 0.06 f	5.65 ± 0.30 g	5.18 ± 0.29 d	1.75 ± 0.11 de	1.37 ± 0.07 def
	1	2.30 ± 0.11 bc	1.91 ± 0.06 de	1.58 ± 0.09 ef	1.53 ± 0.09 ef	8.33 ± 0.48 fg	7.60 ± 0.46 d	1.79 ± 0.09 e	1.51 ± 0.05 cd
	2	2.28 ± 0.09 bc	2.22 ± 0.08 c	1.78 ± 0.08 ef	2.48 ± 0.16 de	10.00 ± 0.51 ef	13.78 ± 0.81 c	1.69 ± 0.08 de	1.68 ± 0.06 bc
	3	2.41 ± 0.08 c	2.56 ± 0.06 a	2.25 ± 0.11 ce	3.60 ± 0.24 c	12.60 ± 0.55 de	20.68 ± 1.12 e	1.71 ± 0.07 e	1.81 ± 0.05 b

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

** *SE* standard error
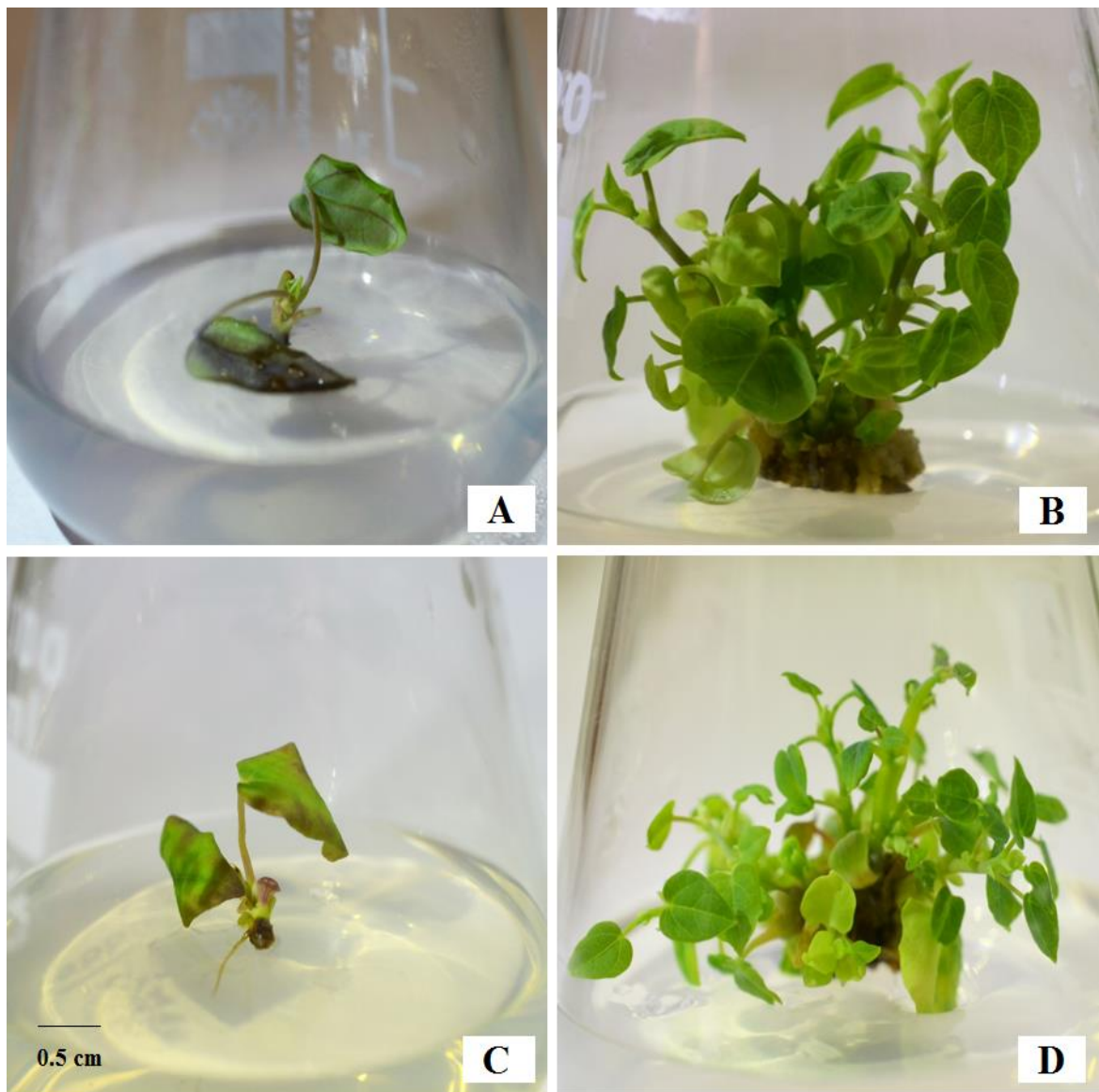
Figure 11: Effect of plant growth regulators on adventitious shoot formation in *Disanthus cercidifolius* after 12 weeks of culture. A – genotype PdS on medium with no PGRs; B – genotype PdS on medium with 2 mg.l^{-1} BAP; C – genotype Truba on medium with no PGRs; B – genotype Truba on medium with 2 mg.l^{-1} BAP. Source: author

Figure 12: Vitrified shoot developed on MS medium supplemented with 3 mg.l^{-1} BAP. Source: author

5.2.1 Histological analysis

Histological investigation provided morphological details on *in vitro* regeneration of plants cultivated on the most efficient medium containing 2 mg.l^{-1} BAP.

Histological analysis showed that new shoots originated from primary explant. Callus was just filling material which enveloped shoots emerging from original explant. Shoots which appeared (seemed to be produced) to be from a callus were associated by vascular bundles with explant (Figure 13). It means that shoots grew from the primary explant and they were just separated by rapid growth of the callus.
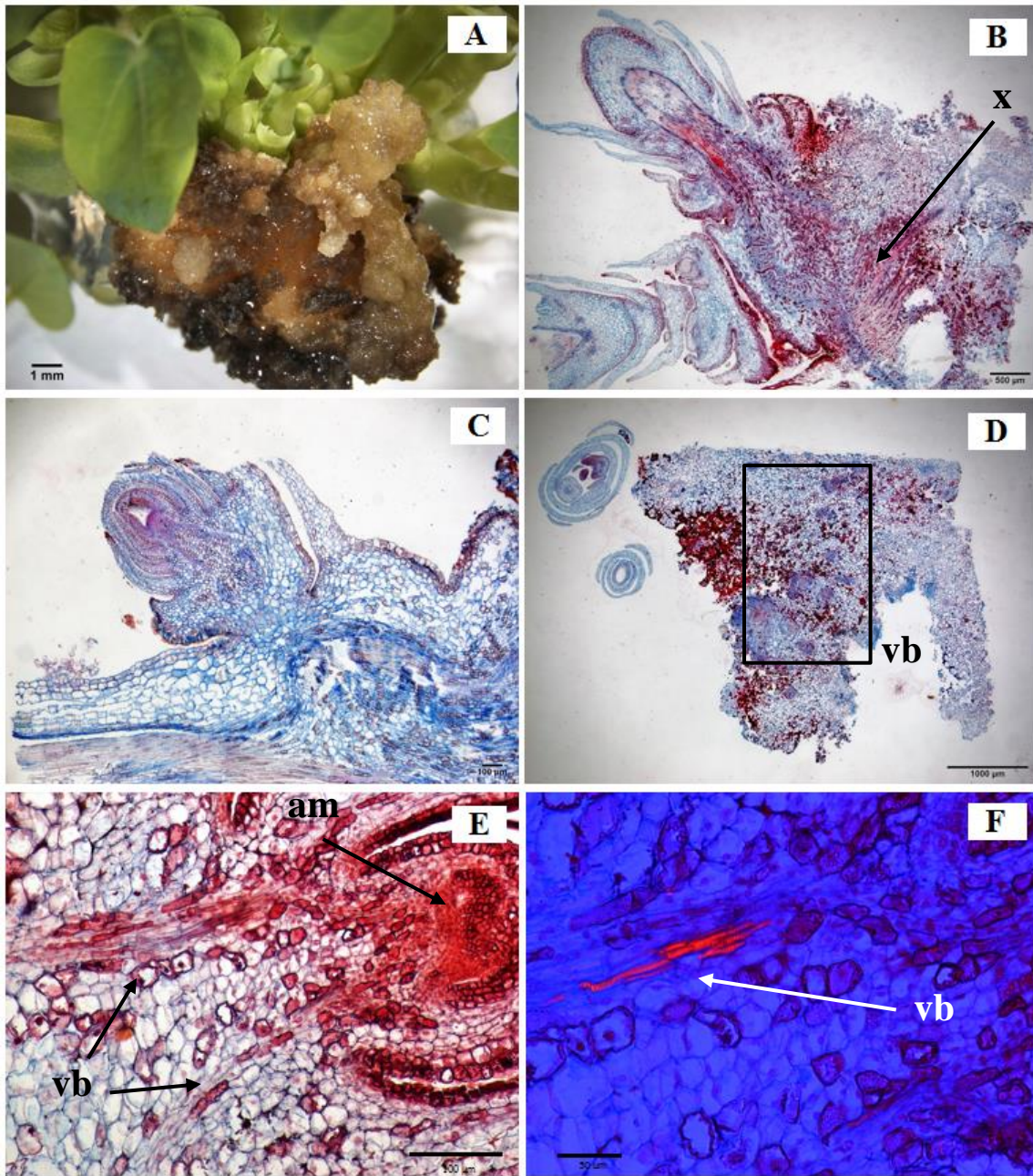
Figure 13: Histological analysis of *in vitro* regenerated plants of *Disanthus cercidifolius*. A – callus surrounding shoots of the plant; B – xylem of primary explant with emerging new shoots with lateral buds; C – growing lateral bud; D – scattered vascular bundles in callus; E – vascular bundles leading from emerging shoot; F – detail of vascular bundle under polarized light; x – xylem; vb – vascular bundles; am – apical meristem. Source: author

5.3   Root induction and *ex vitro* transfer

Plants obtained from the multiplication media did not develop roots (data not shown), therefore shoots from these plants were transferred to rooting media; either half-strength MS medium or half-strength WPM medium with addition of different concentrations of IBA.

Overall, half-strength WPM medium proved to be more effective for root induction of *Disanthus cercidifolius* than did half-strength MS medium (Table 9). Roots started to develop on WPM medium after second week of cultivation whereas root induction on MS medium started after one month. After the 6 weeks of cultivation, the greatest number of roots per explant was produced on a WPM medium containing 0.5 mg.l^{-1} IBA in genotype PdS (in average 3.10 ± 0.53 roots per explant). Although statistically significant differences in number of roots were not detected among this concentration and 0.1 and 0.3 mg.l^{-1} IBA in genotype PdS, plants developed on medium with 0.5 mg.l^{-1} IBA were more vigorous (Figure 14). Plants of the genotype Truba, regardless of IBA concentration, did not developed sufficient number of roots. However plants on medium with addition of 0.5 mg.l^{-1} IBA had rather better and more vigorous growth compared to other treatments.

Figure 14: Rooted shoot of *Disanthus cercidifolius* cultivated on half-strength WPM medium supplemented with 0.5 mg.l^{-1} IBA. Source: author

Table 9: Effect of auxin IBA and type of medium on *in vitro* rooting in two genotypes (PdS, Truba) of *Disanthus cercidifolius* after 6 weeks of culture

Medium	IBA (mg.l^{-1})	Number of roots per explant (mean ± SE)		Root length (cm) (mean ± SE)	
		PdS	Truba	PdS	Truba
½ MS	0	0.10 ± 0.07 c	0.35 ± 0.18 ab	0.70 a	0.70 ± 0.15 a
½ MS	0.1	0.05 ± 0.05 c	0.10 ± 0.07 ab	1.00 a	0.55 ± 0.05 a
½ MS	0.3	0.00	0.10 ± 0.07 ab	0.00	0.40 ± 0.10 a
½ MS	0.5	0.10 ± 0.10 c	0.00	0.70 ± 0.30 a	0.00
½ WPM	0	1.20 ± 0.41 bc	0.60 ± 0.22 ab	1.15 ± 0.13 a	0.67 ± 0.10 a
½ WPM	0.1	2.00 ± 0.31 ab	0.80 ± 0.19 b	1.24 ± 0.10 a	0.66 ± 0.10 a
½ WPM	0.3	1.95 ± 0.37 ab	0.65 ± 0.23 ab	1.27 ± 0.09 a	0.86 ± 0.13 a
½ WPM	0.5	3.10 ± 0.53 a	0.75 ± 0.25 b	1.14 ± 0.08 a	0.72 ± 0.12 a

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

** *SE* standard error

A total of 45 well rooted shoots, each with at least two roots that were minimally 0.5 cm in length, were transferred *ex vitro*. Since genotype Truba did not provide satisfactory results in terms of number of roots, just 15 plants out of 45 all plants were from this genotype. The survival rate of plants after one month of culture in a glasshouse reached 60% in genotype PdS and 33.3% in genotype Truba. The plants grew continuously, no morphological abnormalities were observed and new leaves were produced under *ex vitro* conditions (Figure 15).
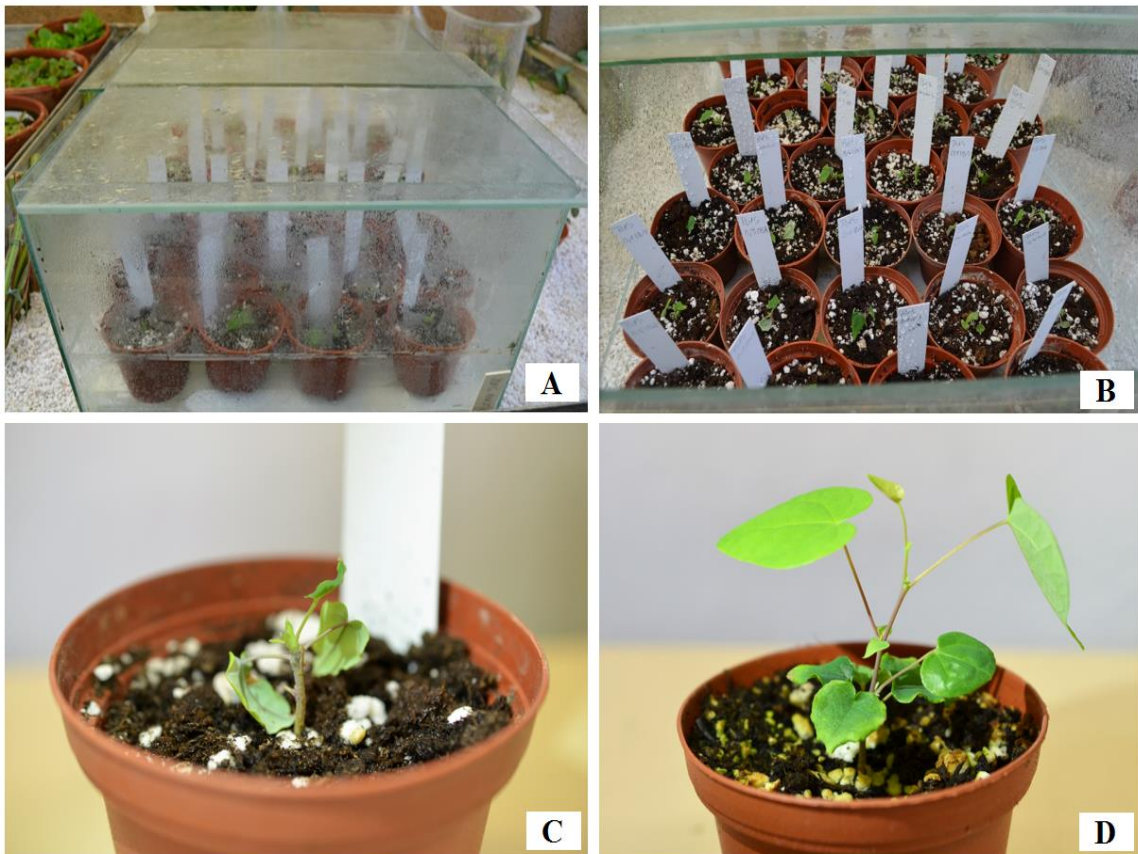
Figure 15: *Ex vitro* transfer of *D. cercidifolius*. A, B – plants in flower pots placed in the glasshouse; C – plant immediately after *ex vitro* transfer; D – plant after one month of cultivation in the glasshouse. Source: author

5.4 DNA extraction and ISSR analysis

The DNA was successfully extracted from the 20 samples out of which 18 were regenerated shoots obtained from the best treatment for *in vitro* propagation and two were the control. Extracted DNA was used for verification of genetic stability of regenerated shoots by ISSR primers. The number of scorable and clear bands per primer varied from two to seven (Table 10). Thirty-nine amplification fragments were generated from all samples, and fragment sizes ranged from 250 to 1300 base pairs. The amplified products of all 10 ISSR primers were monomorphic across *in vitro* regenerants and control plant indicating no genetic variation among samples. Representative monomorphic amplification patterns obtained with ISSR primer 'UBC 826' are shown in Figure 16.

Table 10: List of ISSR primers, annealing temperatures, numbers and sizes of the amplified fragments used for detecting the genetic stability in regenerants of *Disanthus cercidifolius*. T – genotype Truba; P – genotype PdS; bp – base pairs

No.	Primer code	Annealing temperature (°C)	Total no. bands amplified	No. scorable bands per primer	No. and frequency of polymorphic bands per primer	Range of amplification (bp)
1.	'UBC812'	53	70	3 (T) 4 (P)	0 (0%)	350 – 800
2.	'UBC813'	49	40	2	0 (0%)	400 – 800
3.	'UBC814'	49	60	3	0 (0%)	400 – 1000
4.	'UBC824'	54	80	4	0 (0%)	250 – 750
5.	'UBC826'	54	130	6 (T) 7 (P)	0 (0%)	250 – 900
6.	'UBC828'	49	80	4	0 (0%)	350 – 800
7.	'UBC829'	51	70	3 (T) 4 (P)	0 (0%)	700 – 1300
8.	'UBC836'	51	90	4 (T) 5 (P)	0 (0%)	200 – 850
9.	'UBC841'	51	80	4	0 (0%)	250 – 600
10.	'UBC844'	53	80	4	0 (0%)	450 – 1200
Total	–	–	780	39	0 (0%)	–
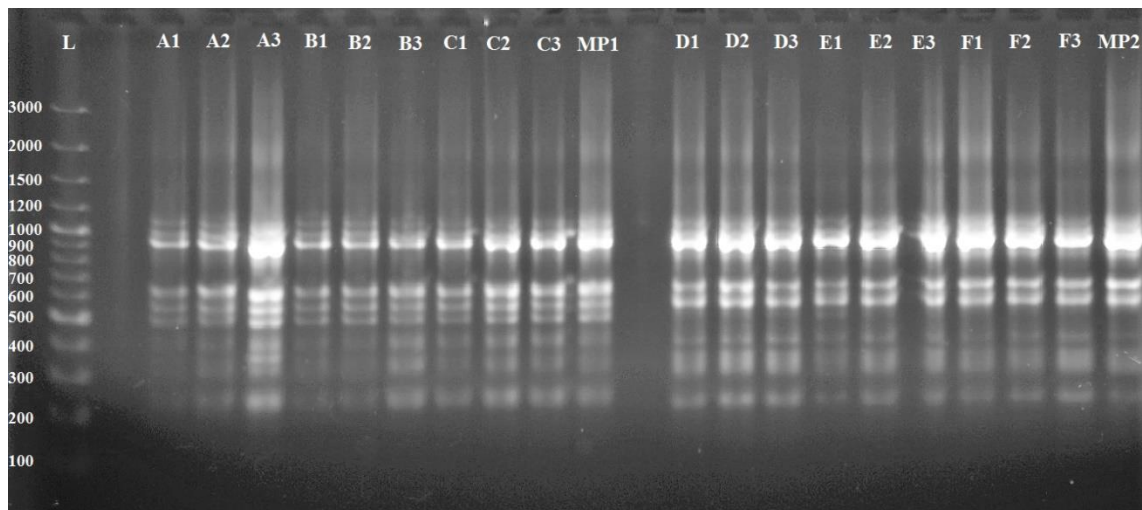
Figure 16: ISSR profile of *in vitro* regenerants and control plant of *D. cercidifolius* using primer 'UBC 826'. L – 100 bp DNA ladder; A-C – *in vitro* regenerated plants from genotype Truba (numbers indicate shoots); MP1 – mother plant (genotype Truba); D-F – *in vitro* regenerated plants from genotype PdS (numbers indicate shoots); MP2 – mother plant (genotype PdS). Source: author

5.5   Analysis of ploidy level

Genetic stability in terms of ploidy level uniformity of *in vitro* regenerants was assessed with flow cytometry in 20 randomly chosen samples (18 from *in vitro* regenerated plants and two from mother plants). Linear histograms of relative nuclear content showed two peaks in all cases (Figure 17); the first peak represented nuclei in the G0/G1 phase of the cell cycle belonging to *Disanthus cercidifolius* sample and the second peak corresponded to nuclei of the internal standard (*Bellis perennis*) in the G0/G1 phase. The DNA-ratios of *in vitro* regenerants of *D. cercidifolius* varied from 0.673 to 0.682 in genotype PdS and from 0.675 to 0.688 in genotype Truba, showing that there were no significant differences to that of the control plant (0.677 in PdS and 0.686 in Truba) and thus suggesting no changes in ploidy level among regenerants.
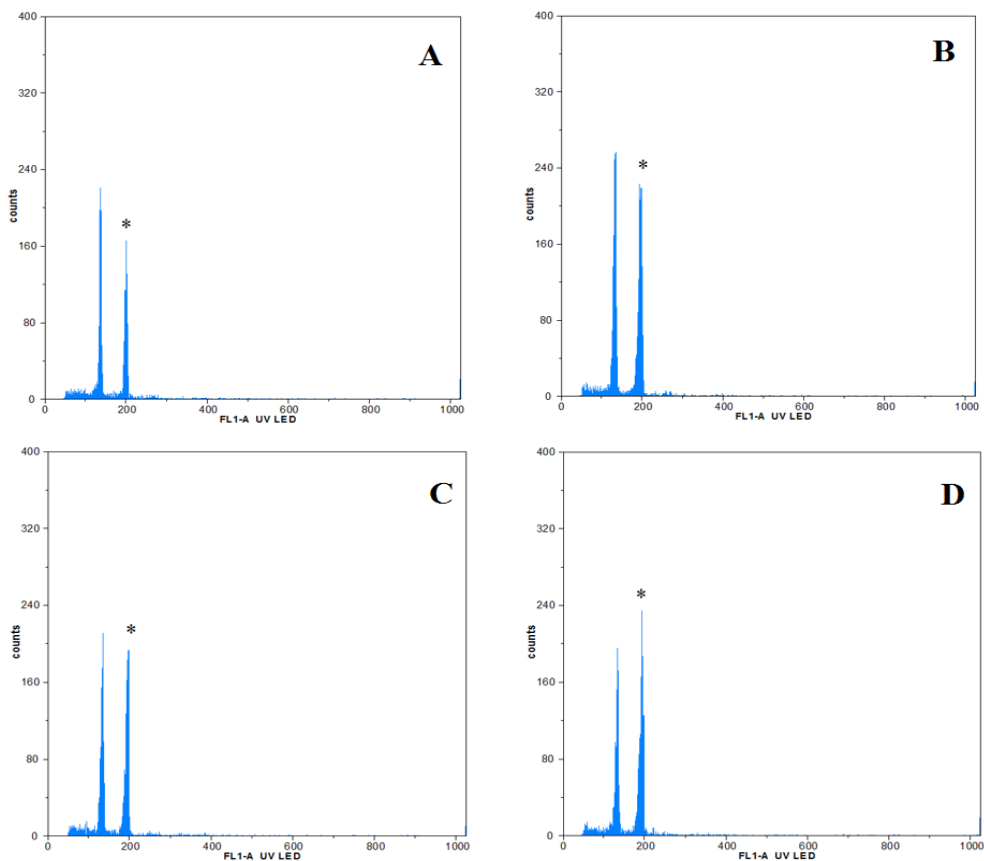
Figure 17: Representative flow cytometric histograms documenting the DNA content of *in vitro* regenerants of *D. cercidifolius*. A – randomly selected *in vitro* regenerant PdS; B – control plant PdS; C –randomly selected *in vitro* regenerant Truba; D – control plant Truba. The peak indicated as "*" correspond to the internal standard (*Bellis perennis*). Source: author

6 Discussion

6.1 Establishment of *in vitro* culture

The use of 0.5% $HgCl_2$ proved to be very effective agent in contamination control during establishment of the culture of *Disanthus cercidifolius*. Studies on species taxonomically close to *Disanthus cercidifolius* such as *Paeonia suffruticosa* (Beruto et al., 2004), *Liquidambar styraciflua* (Ďurkovič and Lux, 2010), *Parrotiopsis jacquemontiana* (Hussain et al., 2012) and *Distylium chinense* (Li et al., 2014) recommended the use of $HgCl_2$ for surface sterilization of plant material as well. In contrast to our experiment, lower concentration of $HgCl_2$ (0.1%) was used for surface sterilization of these species. Beruto et al. (2004) sterilized initial material of *Paeonia suffruticosa* successfully by combination of 0.5% $HgCl_2$ and 1% NaClO.

On the other hand, several authors promote the use of NaClO for surface sterilization of plant material. Konno et al. (2010) sterilized plant material of *Distylium racemosum* (Hamamelidaceae) by 10% NaClO for 20 minutes. Fu et al. (2012) and Wen et al. (2016) also used NaClO for surface sterilization of *Cercidiphyllum japonicum* (Cercidiphyllaceae) and *Paeonia suffruticosa*, but concentration of NaClO as well as duration of sterilization were lower.

As many articles, this study showed that mercuric chloride is an effective agent for surface sterilization of plant material and can be used likewise NaClO.

6.2 *In vitro* propagation

The success in plant tissue culture technology is related to selection of the appropriate culture medium (Ponmurugan and Kumar, 2011). Therefore, the choice of proper plant growth regulators is crucial. In this study, the use of the MS medium supplemented with intermediate concentration of BAP (2 mg.l^{-1}) provided the best results in terms of multiplication rate in both genotypes of *D. cercidifolius*.

This cytokinin had been reported to be optimal for shoot induction in many species botanically related to *Disanthus cercidifolius*, for example, *Liquidambar orientalis* (Erdag and Emek, 2005; Bayraktar et al., 2015), *Liquidambar styraciflua* (Ďurkovič and

Lux, 2010), *Parrotiopsis jacquemontiana* (Hussain et al., 2012), *Cercidiphyllum japonicum* (Fu et al., 2012) and *Paeonia suffruticosa* (Wen et al., 2016). In contrast to our experiment, lower concentrations ranging from 0.7 mg.l^{-1} to 1.1 mg.l^{-1} BAP in combination with small amount of auxin were more effective in shoot proliferation of most these species. The use of intermediate concentration of BAP was recommended in study by Erdag and Emek (2005). They achieved the best results using WPM medium supplemented with 2.5 mg.l^{-1} BAP and 0.1 mg.l^{-1} NAA. Differences in efficiency of various concentrations of BAP can be caused by the fact that the plants belong to different genera and despite taxonomically relatedness, various species/genera might have various cultivation and physiological requirements.

The effect of zeatin as an alternative cytokinin to BAP on induction of adventitious shoots had been also tested in many studies. For many woody plants from various botanical families, for example, for *Arbutus × andrachnoides* (Bertsouklis and Papafotiou, 2011) or *Acer saccharinum* (Preece et al., 1991), zeatin represented a much more efficient solution than other cytokinins. Nevertheless, in *D. cercidifolius*, zeatin was observed to be less effective in the formation of adventitious shoots when compared to BAP. A similar trend was also achieved by Moon et al. (2002) during their research on micropropagation of *Corylopsis coreana* from family Hamamelidaceae. Based on their results, the highest shoot proliferation was obtained on MS medium supplemented with 0.5 up to 3 mg.l^{-1} zeatin and 0.2 mg.l^{-1} BAP, but sole zeatin did not provide satisfactory results.

6.3   Rooting and *ex vitro* transfer

The influence of IBA and two types of basal media was examined in this study. Although MS medium provided excellent results in terms of *in vitro* propagation, half-strength MS medium was not successful in case of *in vitro* rooting and half-strength WPM medium gave better results. The best rooting was achieved on WPM medium with 0.5 mg.l^{-1} IBA in genotype PdS, whereas plants of the genotype Truba, regardless of IBA concentration, did not developed sufficient number of roots.

Efficiency of WPM medium for root induction had been confirmed in many studies on species taxonomically related to *Disanthus cercidifolius*, for example, on *Paeonia*

suffruticosa (Beruto et al., 2004) *Liquidambar orientalis* (Erdag and Emek, 2005; Bayraktar et al., 2015) and *Liquidambar styraciflua* (Ďurkovič and Lux, 2010). On the other hand, some authors recommended the use of MS medium for rooting like Hussain et al. (2012) in research on *Parrotiopsis jacquemontiana*.

IBA provided satisfactory results in terms of root induction in our study, especially in genotype PdS. The use of this auxin had been reported in many studies, such as in those focused on micropropagation of *Paeonia suffruticosa* (Beruto et al., 2004), *Liquidambar orientalis* (Erdag and Emek, 2005; Bayraktar et al., 2015) and *Parrotiopsis jacquemontiana* (Hussain et al., 2012). In contrast to our experiment, higher concentrations ranging from 2 mg.l^{-1} to 4 mg.l^{-1} IBA were more effective in root induction of *Liquidambar* spp. (Erdag and Emek, 2005; Bayraktar et al., 2015). Hussain et al. (2012) compared the effect of auxins IBA, NAA and IAA on root formation of *Parrotiopsis jacquemontiana*. According to their research, IBA is the most suitable for *in vitro* rooting of plants of family Hamamelidaceae. According to Beruto et al. (2004), IBA improves root formation of tree peony as well, but it is not really necessary provided the shoots were pre-treated at 2 °C for 7 days.

Though many authors suggest the use of IBA, some studies showed the efficiency of NAA for root induction, e.g., in *Corylopsis coreana* (Moon et al., 2002), *Liquidambar styraciflua* (Ďurkovič and Lux, 2010) and *Cercidiphyllum japonicum* (Fu et al., 2012). For instance, Fu et al. (2012) compared the effect of NAA and IBA, which resulted in better rooting with 0.5 mg.l^{-1} NAA.

This study proved that rooting is genotype dependent, because genotype PdS rooted better than genotype Truba. It is quite common phenomenon which was also achieved by Monier and Ochatt (1995) in the study on micropropagation of *Cotoneaster* spp. where five genotypes were tested.

Successful management of plant transfer to *ex vitro* conditions is very important in terms of economy since amounts of chemicals, labour and energy were invested in micropropagation (Pavlová, 1992). In our experiment, for *ex vitro* transfer, a sterilized mixture of garden substrate:peat:perlite (1:1:1) was used and survival rate of plants after one month of culture reached 60% in genotype PdS and 33.3% in genotype Truba. Although, some studies reported relatively high survival rate (80 – 95%) after *ex vitro*

transfer (Erdag and Emek, 2005; Li et al., 2014), several species taxonomically related to *D. cercidifolius* had been reported to have poor survival rates (40 – 67%) after *ex vitro* transfer (Moon et al., 2002; Hussain et al., 2012; Wen et al., 2016) indicating that this group of plants might be quite susceptible during acclimation process. Survival rates in plants transferred to *ex vitro* conditions could be improved in many ways, for instance based on results of Wen et al. (2016), plantlets which were inoculated by arbuscular mycorrhizal fungi *Glomus mosseae* showed a high survival rate and vigorous growth in comparison with uninoculated plants. As in this study on *D. cercidifolius*, differences in survival rate between different genotypes were achieved similarly in study on *Paeonia suffruticosa* (Wen et al., 2016).

6.4   Assessment of genetic fidelity of regenerants

Changes in DNA sequence and in DNA ploidy level are among the most frequent genetic variations found in *in vitro* cultures (Brito et al., 2010). Therefore, in our study, genetic stability of regenerated plantlets was assessed using ISSR primers and flow cytometry, after *in vitro* propagation.

Since occurrence of somaclonal variation is widely associated to regeneration of plants from callus (Karp, 1995) and in our experiments, callus developed on the basis of the original explant, histological analysis was carried out to reveal morphological origin of new shoots. Histological analysis proved that new shoots of *Disanthus cercidifolius* regenerated from primary explant, and not from callus. It means that micropropagation was carried out via direct morphogenesis. Direct morphogenesis is preferred for mass micropropagation of plants since direct shoot regeneration from explants usually maintains genotype fidelity (Ghimire et al., 2012). This assumption was confirmed in our study as no genetic variability among regenerants of *D. cercidifolius* was detected.

ISSR markers were found to be a reliable method for the genetic analysis of *in vitro* regenerants in many species. They had been successfully used to assess genetic stability in many woody species such as *Ochreinauclea missionis* (Chandrika and Rai, 2009), *Platanus acerifolia* (Huang et al., 2009) and *Eucalyptus camaldulensis* (Shanthi et al., 2015). Genetic stability using ISSR markers had been proven in many micropropagated

woody plants. Genetic fidelity was detected in *Salvadora oleoides* (Phulwaria et al., 2014), *Moringa peregrina* (Al Khateeb et al., 2013), *Pithecellobium dulce* (Goyal et al., 2012) and *Terminalia bellerica* (Dangi et al., 2014). Nevertheless, somaclonal variation sometimes occurs after *in vitro* cultivation. Ahmed et al. (2012) detected genetic variation between the regenerated plantlet and its corresponding callus in *Phoenix dactylifera* using ISSR analysis.

Flow cytometric analysis was carried out in order to reveal the potential occurrences of genomic changes and the results confirmed ploidy stability of *in vitro* regenerants. Many studies on woody plants proved unchanged ploidy level in regenerants after micropropagation, as in *Juniperus phoenicea* (Loureiro et al., 2007) and *Olea* spp. (Brito et al., 2010), as well as in plants after somatic embryogenesis, as in *Quercus suber* (Loureiro et al., 2005). Nevertheless, ploidy variations can occur after somatic embryogenesis and after long term *in vitro* cultivation of woody species as showed studies on *Coffea arabica* (Clarindo et al., 2012) and *Larix decidua* (Von Aderkas et al., 2003). Thus, it is highly advisable to complete each micropropagation protocol with a reliable system of assessment of *in vitro* regenerated plants to determine the success of a protocol.

7 Conclusion

This study provides first report on the micropropagation of *Disanthus cercidifolius*, a shrub with great ornamental potential. *In vitro* culture was successfully established using sprouting buds from two mature shrubs and 0.5% $HgCl_2$ as sterilization agents. For *in vitro* propagation, 2 mg.l^{-1} BAP was the most efficient treatment providing highest number of shoots per explant as well as number of buds per explant in both *D. cercidifolius* genotypes. On the contrary, zeatin did not proved to be suitable cytokinin for multiplication of this species. Histological analysis of explants from the most efficient medium showed that new shoots developed from primary explant despite formation of callus on the basis of the original plant material. The new shoots were successfully rooted on WPM medium with 0.5 mg.l^{-1} IBA. After *ex vitro* transfer, the survival rate of plants reached 60% in genotype PdS and 33.3% in genotype Truba indicating that whole micropropagation process is genotype dependent. Finally, genetic stability of *in vitro* regenerants was confirmed by ISSR analysis and flow cytometry.

In conclusion, the protocol described here could be utilized for effective mass propagation of *Disanthus cercidifolius* for commercial as well as for conservation purposes.

7.1 Recommendation

Within further research, due to lower percentage of plant survival after *ex vitro* transfer and rooting of microshoots, especially in genotype Truba, testing more rooting media can be recommended. Higher concentrations of IBA or other types of auxins at various concentrations should be used to improve both, rooting and *ex vitro* transfer. For *ex vitro* transfer, various substrates composed of different materials at various rates should be tested. Protocol for micropropagation, optimized in this study for two genotypes, could be also verified for various genotypes.

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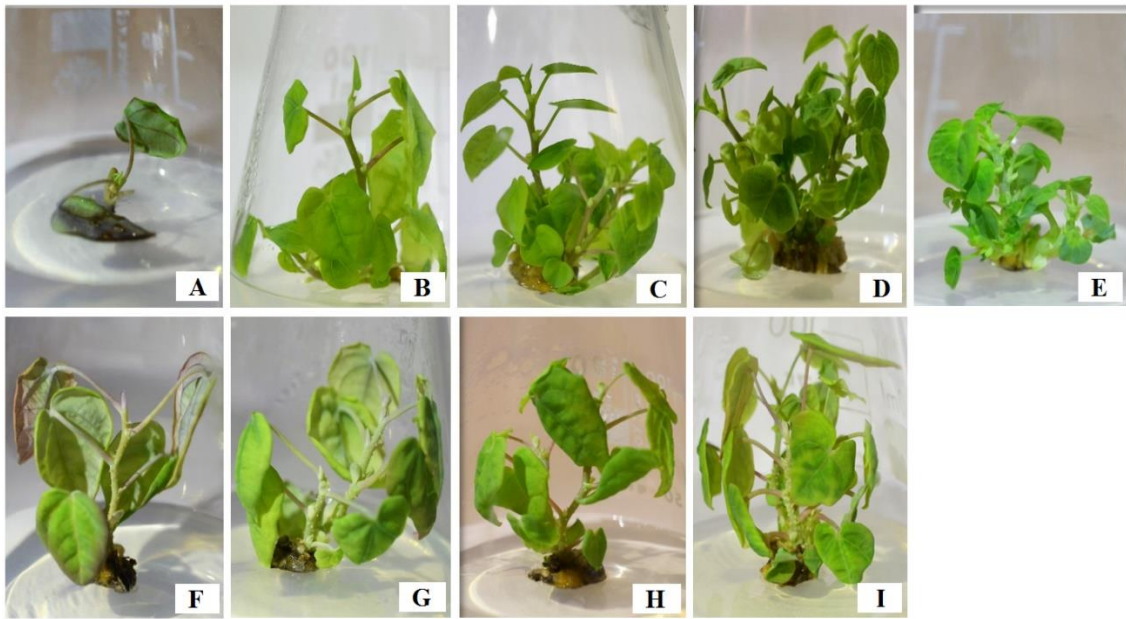
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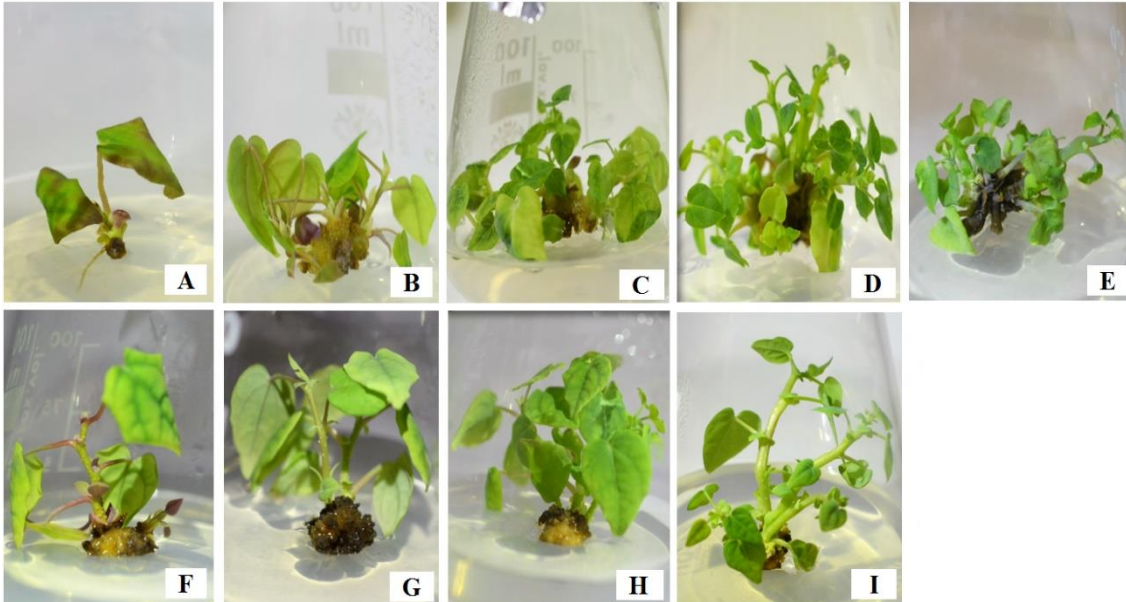
Appendices

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I

Figure A: Effect of plant growth regulators on adventitious shoot formation in *Disanthus cercidifolius* in all treatments. Genotype PdS cultivated on MS medium supplemented with: A – no PGRs; B – 0.5 mg.l^{-1} BAP; C – 1 mg.l^{-1} BAP; D – 2 mg.l^{-1} BAP; E – 3 mg.l^{-1} BAP; F – 0.5 mg.l^{-1} ZEA; G – 1 mg.l^{-1} ZEA; H – 2 mg.l^{-1} ZEA; I – 3 mg.l^{-1} ZEA. Source: author

Figure B: Effect of plant growth regulators on adventitious shoot formation in *Disanthus cercidifolius* in all treatments. Genotype Truba cultivated on MS medium supplemented with: A – no PGRs; B – 0.5 mg.l^{-1} BAP; C – 1 mg.l^{-1} BAP; D – 2 mg.l^{-1} BAP; E – 3 mg.l^{-1} BAP; F – 0.5 mg.l^{-1} ZEA; G – 1 mg.l^{-1} ZEA; H – 2 mg.l^{-1} ZEA; I – 3 mg.l^{-1} ZEA. Source: author