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***In vitro* propagation and conservation of *Adenophora liliifolia* Ledeb. ex A. DC., an  
endangered and potential ornamental species**

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## ABBREVIATIONS

MS	Murashige and Skoog medium (Murashige and Skoog, 1962)
CW	Coconut water
AgNO <sub>3</sub>	Silver nitrate
PGR	Plant growth regulator
IBA	Indole-3-butyric acid
NAA	1-Naphthaleneacetic acid
BAP	6-Benzylaminopurine
GA3	Gibberellic acid
IAA	Indole-3-acetic acid
KIN	Kinetin
Fe-EDTA	Ferric ethylenediaminetetraacetic acid
MANOVA	Multivariate analysis of variance

## I. INTRODUCTION

*In vitro* culture and cryopreservation are instruments of biotechnology cooperating in the conservation of endangered species (Paunescu, 2009). In addition, molecular markers are effective and reliable tools for the analysis of genome architectures and gene polymorphisms which have been used for various gene bank management practices, including utilization and characterization of the germplasm material (Barcaccia, 2009; Börner *et al.*, 2012).

The propagation and conservation of endangered plants with ornamental potential can take advantage of these biotechnological tools. Datta (2019) claims that plants with high floriculture importance could be preserved using *in vitro* culture. Thus, a horticultural system will protect endangered species from extinction.

The floriculture industry is always looking for new materials and novelties (Kováts & Karip-Szabó, 2003). *Adenophora liliifolia* (L.) Ledeb. ex A. DC. (Király, 2009), an endangered species from Hungary and other Central European countries, is characterized by its showy and colorful flowers and herbaceous and perennial plant structure. However, the extinction of its habitats, which are suitable for its growth and reproduction, is the leading cause of the threat of this species (Čepelová & Prausová, 2017).

The protection of the natural populations of *A. liliifolia* is necessary. Paunescu (2009) recommends establishing *in vitro* plant stock of wild populations, enabling population recovery, ecological studies, economic uses, or molecular investigations.

This project focuses on the germplasm preservation of *A. liliifolia*, establishing a protocol for *ex situ* conservation through an active collection used in future experiments to repopulate the original habitat. Finally, the conservation of *A. liliifolia* through its ornamental use could preserve the species as a long-term ornamental crop.

## II. OBJECTIVE

This work aims to:

- 1) Demonstrate that *Adenophora liliifolia* can be multiplied, rooted, and acclimatized on synthetic medium, Murashige and Skoog (MS) supplemented with different doses of other plant growth regulators (PGR) and natural extracts, with good efficiency.
- 2) Study the effects of different PGR on the development of micropropagated *Adenophora liliifolia* plants.
- 3) Determine a suitable growing medium for *Adenophora liliifolia*.
- 4) Establish a simple and rapid micropropagation system.
- 5) Generate *in vitro* plantlets for future reintroduction purposes.

Specific objectives:

- 1) To identify the adequate doses of Silver Nitrate ( $\text{AgNO}_3$ ) in *in vitro* culture.
- 2) To identify the adequate doses of 1-Naphthaleneacetic acid (NAA) in *in vitro* culture.
- 3) To identify the adequate doses of coconut water (CW) in *in vitro* culture.
- 4) To identify the adequate pH level in *in vitro* culture.
- 5) To identify the Chlorophyll a + b and Carotenoids content values in samples of pH gradient treatment and samples of different doses of CW.
- 6) To identify the adequate acclimatized option using covered or uncovered plants with agronet and different types of substrates.

### III. LITERATURE REVIEW

#### III.1. Biodiversity

“Biological diversity (often shortened to ‘biodiversity’) measures the variation in genes, species, and ecosystems. It is valuable because diversity is the base of the stability and sustainable functions of natural systems; of its enormously wide range for potential and unexplored uses; there is evidence that removal of ecosystem components can have negative impacts; variety is inherently interesting and more attractive” (EEA, 2016).

##### III.1.1. European biodiversity

Europe contains 11,500 (around 3.6 %) of the estimated 320,000 vascular plant species globally (Mutke *et al.*, 2010).

“Landlocked Hungary lies in the central part of the Danube Basin. Although not a very large country, Hungary is quite rich in biodiversity with more than 2,000 plant species, about 400 bird species, 87 mammal species, etc. The country has 10 national parks, 145 minor nature reserves, and 35 landscape protection areas. The Aggtelek and Hortobágy National Parks are also listed on the UNESCO World Heritage Site” (IUCN, 2021a).

##### III.1.2. Native plants with ornamental potential

Floriculture and the production of ornamental plants is one of the most diverse sectors of horticulture (Volckaert & Gobin, 2010). Floriculture bases its industry on novelties, and introducing new ornamental crops into commercial production develops this sector (Karlović, 2009).

Native plants are a source of new material for the industry and represent an unexploited origin of great potential value (Karlović, 2009; Yilmaz & Yilmaz, 2009).

Some native Hungarian plants with high value as ornamentals are *Sorbus* species (Farkas, 1999), *Fraxinus ornus* L., and *Fraxinus angustifolia* Vahl subsp. *danubialis* Pouzar (FRAXIGEN, 2005; Király, 2009). Moreover, other Hungarian wild plants such as the dwarf *Alcea* group, the *Alcea* x *Althaea* hybrids, *Limonium*, *Cichorium*, and lawn grass species are introduced into ornamental plants breeding programs (Kováts & Karip-Szabó, 2003).

To refer to the application of the techniques and knowledge base of horticulture to the conservation of rare and threatened plants, Affolter (1997) defined the term “conservation horticulture”. Conservation horticulture knowledge develops horticultural innovations in growing plants, which helps species be saved

from wild extinction (Gratzfeld, 2017). Effective conservation horticulture must consider capturing genetic diversity, data stewardship, cultivation, and propagation (Kay *et al.*, 2011).

Additionally, biotechnological techniques are tools to maintain the variability of autochthonous species. It could represent progress towards long-term preservation and introduction into the floriculture industry of species with ornamental potential (Seglie *et al.*, 2012). An example of this is Kiani *et al.* (2010) and Kiani *et al.* (2013) experiments, which established the conservation of *Colutea gifana* Parsa and *Diaphanoptera khorasanica* Rech.f., rare and potential ornamental species with limited reproductive capacity, using *in vitro* method. The main results of these researchers were micropropagation protocols for large-scale multiplication under *ex situ* and *in situ* conservation using single node explants.

### III.2. *Campanulaceae* family

The *Campanulaceae* Jussieu, nom. cons. family has a worldwide distribution (Simpson, 2010). This family comprises 86 genera and more than 2,300 species in numerous habitats, from arctic to tropical rainforests (Deyuan *et al.*, 2011; Crowl *et al.*, 2016). It belongs to the Asterids, a major angiosperm clade, and the Order Asterales (Bilz, 2011; Stull *et al.*, 2018; Roskov *et al.*, 2019). The family includes five major lineages: *Campanuloideae* Burnett, *Lobelioideae* Burnett, *Cyphioideae* Schönland, *Nemacladoideae* M. H. G. Gustafsson, and *Cyphocarpoideae* Gustafsson (Stevens, 2001; Crowl *et al.*, 2016; Yoo *et al.*, 2018). *Campanuloideae* is divided in three tribes: *Cyanantheae* Meisner, *Wahlenbergieae* Endlicher, and *Campanuleae* Dumortier (Stevens, 2001).

The *Campanulaceae* are mostly perennial herbs (often with rhizomes or caudices), shrubs, or trees. Additionally, the family consists of hermaphroditic (dioecious in case of *Dialypetalum* Benth. genus) flowers, berry or capsule fruits, and seeds with starchy endospermous (Stevens, 2001; Simpson, 2010; Deyuan *et al.*, 2011).

This family exhibits racemose inflorescence; flowers actinomorphic or zygomorphic; flowers large, (3-)5(-10)-merous, monosymmetric. Petals are fused into a corolla with 3 - 8 lobes. Flowers may be star- or bell-shaped in *Campanuloideae*, while tubular and bilaterally symmetric in most *Lobelioideae* (Stevens, 2001; Simpson, 2010).

The economic importance of the family comprises local medicinal uses and cultivated ornamental species, for instance, the *Adenophora*, *Campanula*, *Lobelia* genus (Simpson, 2010). However, the IUCN (2021b) exposes 18 Extinct species, 2 Extinct In The Wild species, 72 Critically Endangered species, 79 Endangered species, 51 Vulnerable species, 12 near-threatened species, and 31 Least Concern species from the *Campanulaceae* family.



*Campanula polymorpha* Witas., an endemic plant of the Carpathian Mountains (Paunescu, 2010), *Campanula primulifolia* Brot., a critically endangered species in the Iberian Peninsula (Trias-Blasi *et al.*, 2011), and *Musschia isambertoii* M.Seq., R.Jardim, Magda Silva & L.Carvalho, a critically endangered and endemic plant from Madeira Archipelago in Portugal (Menezes de Sequeira *et al.*, 2021) are some examples of threatened *Campanulaceae* species. Furthermore, some endangered species among the *Adenophora* Fischer genus are *Adenophora palustris* Kom., an endangered wetland plant species in Japan (Masumoto *et al.*, 2011), and *Adenophora xiaoxiensis* D.G.Zhang, D.Xie & X.Y.Yi, an endangered species from China that grows in a restricted area (Yi *et al.*, 2019).

### III.2.1. *Adenophora* genus

The genus *Adenophora* comprises about 62 species in eastern Asia, Vietnam, and south to India, with one species extending into Europe and another endemic to Crimea (Deyuan *et al.*, 2011).

*Adenophora* belongs to the tribe *Campanuleae* (Lammers, 2007 as cited in Xu & Hong, 2020). It has been segregated from *Campanula* L. based on the presence of a conspicuous tubular nectariferous disc. *Adenophora* and *Campanula* are sister taxa based on the cpDNA gene order analyses, and *Adenophora*'s chloroplast genome is derived relative to *Campanula*'s (Cosner *et al.*, 2004). Likewise, Cheon *et al.* (2017) studied a whole chloroplastid genome variation analysis in some *Adenophora* spp. and confirmed a more precise molecular work based on 76 protein-coding genes, which shows that *Campanuloids* were divided into two major groups: the *Campanula s. str.* clade and the *Rapunculus* clade.

### III.3. *Adenophora liliifolia*

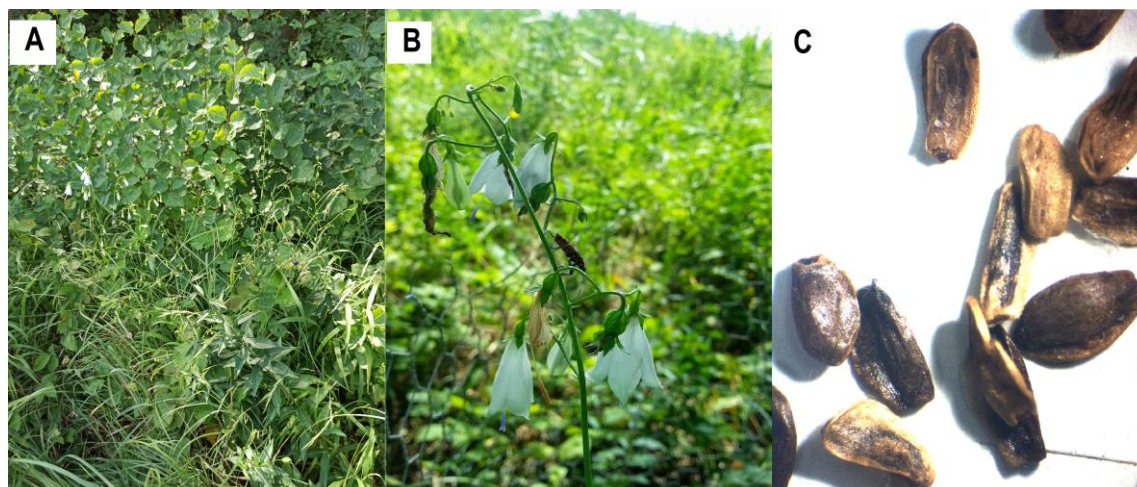
*Adenophora liliifolia*, also well-known as the Lilyleaf ladybell (Bilz, 2011), is sometimes called false campanula (Missouri Botanical Garden, n.d.).

#### III.3.1. Characteristics of the species

Lilyleaf ladybell (Fig.1.) is a long-lived perennial plant with a height of (33-) 40-90 (-206) cm, and overwintering buds located at the soil surface (hemicryptophyte) (Čepelová & Prausová, 2017; Korzeniak & Nobis, 2004, as cited in Rapa, 2017). It has carrot-shaped roots; stems glabrous, branched or simple; shortly petiolate or cauline leaves sessile, ovate or blade lanceolate, both surfaces glabrous, rarely sparsely white hirsute abaxially, margin serrate, apex acute to shortly acuminate; petiole up to 6 mm when is present (Deyuan *et al.*, 2011; Missouri Botanical Garden, n.d.).

The inflorescence is a narrow panicle with branches up to 7 flowers, or rarely only several flowers forming a pseudoraceme; pedicels slender up to 2.5 cm. Hypanthium obovoid or obconic, glabrous; lobes lanceolate, mostly reflexed from anthesis, margin rarely entire or usually with a pair of denticles. The corolla

color is typically blue, lilac-blue, or pale blue, campanulate or bell-shaped with 1.2 to 2.2 cm of size; lobes ovate, ca. 1/2 as long as tube, apex acute which are mildly fragrant. Disk shortly tubular with a size of 1.5–2.5 mm, glabrous. Style slightly exerted or as long as corolla (Deyuan *et al.*, 2011).



**Figure 1.:** A. *liliifolia* plant structure: A. complete plant from Ócsa population growing in natural habitat (photo by the author), B. flowers from Dabas-Hungary population, and C. seeds (photos by Kovács Zsófia).

### III.3.2. Distribution and habitat conditions

*Adenophora liliifolia* grows in the scrub Forests in the far northwest of China, Kazakhstan, and Europe (Deyuan *et al.*, 2011). The distribution of *A. liliifolia* in Europe is extended to Central Europe (Austria, Croatia, Czech Republic, Germany, Hungary, mainland Italy, Poland, Slovakia, and Slovenia), Eastern Europe (Belarus, Romania, Ukraine, and Moldova), Southern Europe (Bosnia and Herzegovina, Montenegro, and Serbia), Western Europe (Switzerland) and Central, South and East European Russia (Bilz, 2011).

Prausová *et al.* (2016) suggest a similar genetic background for most populations of *Adenophora liliifolia* from the Czech Republic, Poland, and Slovakia. Differing from the two populations of Central and South Slovakia, South Hungary, and Romania, they were isolated for a more extended period and showed more evident genetic differentiation.

Lilyleaf ladybell is considered a “species indicator of thermophilous forest hotspots signaling remnant pools of biodiversity” (Kiedrzyński & Jakubowska-Gabara, 2014).

This species is located in Euro-Siberian steppic woods and Pannonian woods with *Quercus pubescens* Willd., *Quercus petraea* (Matt.) Liebl., and *Carpinus betulus* L., oak-hornbeam forests (Galio-Carpinetum), alluvial forests of Alno-Ulmion communities, Asperulo-Fagetum beech forests, Medio-European limestone beech forests of the Cephalanthero-Fagin, open pine forests, and forest margins. Also, it has been reported from intermittently wet Molinia meadows on calcareous, peaty, or clayey-silt-laden soils (Bilz, 2011).

*Adenophora liliifolia* is called “csengettyűvirág” in Hungarian (EPPO, 2020). It is found in altitudes between 100 to 680 m. This plant is localized in the Riparian mixed gallery forest in Ócsa, Kiskőrös, and Dabas; their bedrocks are characterized as organic-rich sediment, lacustrine and paludal clay, silt, calcareous mud, peat. Intermittently wet *Molinia* meadows in Regéc and Füzér with rhyolite and andesite as bedrocks and the mountain hay meadows in Aggtelek with light steinalm limestones are also habitats of *A. liliifolia* (Fig.2.) (Prausová *et al.*, 2016).



**Figure 2.:** Lily-leaf ladybell and its presence in Hungary (Árgay *et al.*, 2018).

Bilz (2011) mentions 180 individuals with a stable population trend in Hungary over the last ten years. However, three from more than ten known localities have become extinct in the last 50 years. Farkas (2020) points out Dabas and Ócsa populations individual numbers have strongly decreased in the last decades.

*Adenophora liliifolia* flowers are characteristic because its showiness and fragrance. Deyuan *et al.* (2011) and Rapa (2017) mention flowering between July to August. Missouri Botanical Garden (n.d.) and Čepelová & Prausová (2017) register the blooming time in May or between July to September. July and August are the main periods in Hungary, but late flowering in September is also usual (Király, 2009).

*Adenophora liliifolia* individual shoots from one cluster might flower at slightly different times, thus prolonging the flowering period. Moreover, during the flowering stage, young specimens could be easily confused with other bellflowers (Deyuan *et al.*, 2011; Rapa, 2017). Lazarski (2017) observes populations ranging from two flowering shoots to more than 50 in Poland.

Numerous insects pollinate the ladybell, and the seeds ripen are dispersed in late August and throughout September (Korzeniak & Nobis, 2004, as cited in Rapa, 2017).

### III.3.3. Cultivation

*Adenophora liliifolia* requires a medium level of water and a medium level of maintenance with no serious insect or disease problems. This species prefers to grow in full sun to partial shade areas. It is easy to grow in an average, medium, well-drained soil rich in organic material and moist alkaline soil (Missouri Botanical Garden, n. d.; Prausová *et al.*, 2016).

Seeds are a way of propagation; nevertheless, plants are somewhat difficult to divide and move once established in the garden. *A. liliifolia* should be left undisturbed for an optimal growing process (Missouri Botanical Garden, n.d.).

The plant can also reproduce vegetatively from fragmented roots (Korzeniak & Nobis, 2004, as cited in Rapa, 2017).

### III.4. Endangered species

Species such as *A. liliifolia* have been influenced by human activity and affected their natural habitat distribution (Prausová *et al.*, 2016). The populations of this species show a decline currently generalized in the whole of Europe (Manole *et al.*, 2015).

Based on IUCN, the *A. liliifolia* population is considered as Least Concern. Therefore, the risk of going extinct is relatively low; nevertheless, various national red lists and The Carpathian List of Endangered Species classed it as threatened because of the affecting of its habitats (Bilz, 2011; Witkowski *et al.*, 2003).

*Adenophora liliifolia* is not only a Critically Endangered species in Hungary (Király, 2007) but also it is included in the group of Critically Endangered plants of Poland (Jackowiak *et al.*, 2007), Critically Endangered plants of the Czech Republic (Grulich, 2012), Vulnerable plants of Slovakia (Turis *et al.*, 2014), Threatened with Extinction plant of Germany (Rote-Liste-Zentrum, n.d.), Near Threatened plant in Italy (Rossi *et al.*, 2013). Likewise, Witkowski *et al.* (2003) reveal *A. liliifolia* is a Vulnerable plant in Romania and Critically Endangered in Austria. The National Red List (n.d.) suggests this plant as Near Threatened plant in Croatia and Endangered in Switzerland.

Lilyleaf ladybell habitats are affected by forest plantations, deforestation, trampling by cattle and overgrazing, abandonment of grazing or mowing, damage by game species and non-native species (Bilz, 2011).

Prausová *et al.* (2016) evidence that the decline of *A. liliifolia* populations in Central Europe is related to eutrophication, shading, overpopulation of wild animals, and expansive broad-leaved herbs. Most of the

damage identified in the population of *A. liliifolia* in the Czech Republic had the form of nibbling by animals (NCA CR, 2017).

Manole *et al.* (2015) mention that one possible cause for the population decline of Lilyleaf ladybell is the overgrowth of shrubby and herbaceous vegetation that impedes seed germination. The reduction of *A. liliifolia* in Europe may be explained because of the level of germination, specific requirements conjugated with habitat fragmentation, and structure alteration.

Vaculná *et al.* (2021) suggest that ongoing climate change will worsen the situation and influence the future distribution of *A. liliifolia* in Europe.

Farkas & Vojtkó (2013) indicate that the main factors for the overall decline of the species in Hungary are the very low rate of flower and fruit production and the sensitivity to grazing and trampling by ungulates.

Bilz (2011) claims adequate measures to prevent further declines of this species.

#### **III.4.1. Protection and Conservation in Europe**

*Adenophora liliifolia* is protected in Europe by EU Habitats Directive, and it is included in the II and IV appendixes of the EU Habitat Directive. Moreover, Natura 2000 sites register 70 protected areas designated for the conservation of this species in Germany (2), Italy (12), Poland (17), Slovakia (15), Czech Republic (5), Romania (9), Hungary (5), Slovenia (2), Austria (2), and Croatia (1) (EEA, 2019).

Furthermore, two basic approaches for conserving plant genetic resources are *ex situ* and *in situ* conservation (Radha *et al.*, 2012). *Adenophora liliifolia* is considered to be part of these two conservation strategies not only in Hungary but also in other countries like Poland, Romania, and the Czech Republic, which are taking actions for the conservation of this species (Pénzes-Kónya *et al.*, 2015; Kapler *et al.*, 2015; Manole *et al.*, 2015; NCA CR, 2017; Kovács & Tillyné, 2019).

#### **III.4.2. *In situ* conservation**

The Convention on Biological Diversity (1992) defines *in situ* conservation as “The conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species in their natural surroundings and, in the case of domesticated or cultivated species, in the surroundings where they have developed their distinctive properties.”

*In situ* approaches encompass genetic reserves, on-farm and home garden conservation (Radha *et al.*, 2012; Malhotra *et al.*, 2019). On the one hand, genetic reserves (gene management zones/units) are a particular kind of reserve where the purpose is the long-term conservation of genetic diversity in wild

populations of target species (Heywood & Dullo, 2005). On the other hand, *in situ* conservation on-farm concerns the entire agroecosystems, including immediately useful species (such as cultivated crops, forages, and agroforestry species) and their wild relatives that may be growing in nearby areas (Jarvis *et al.*, 2000). Additionally, home gardens are microenvironments containing high levels of species and genetic diversity within larger farming systems, which are essential sources of food, fodder, fuel, medicines, spices, construction materials, and income in many countries worldwide (Watson & Eyzaguirre, 2001).

Hungary preserves *A. liliifolia* in Special Areas of Conservation like Északi-Zempléni-hegység (HUBN20085), Központi-Zempléni-hegység (HUBN20084), Aggteleki-karszt és peremterületei (HUAN20001), Turjánvidék (HUDI20051), and Kiskőrösi turjános (HUKN20022) (EEA, 2019).

One problem in the conservation process of this species is the presence of deer. Deer feed on *A. liliifolia*, chewing back its leafy and flowering shoots (Mered'a & Hodálová, 2011, as cited in Farkas, 2020). Deer can cause severe damages to the flora, even producing economic losses in agriculture. There are possible methods to protect plants from deer damage, such as physical barriers or repellents based on odor (Fig. 3.). This last one seems to be the most effective method to reduce deer damage (Mckenna & Woeste, 2014; University of Minnesota, 2018; De las Mercedes *et al.*, 2020)



**Figure 3.:** Protection against deer: A. chemical repellents in Ócsa and Dabas population (photo by the author) B. physical barrier in Dabas (photo by Kovács Zsófia).

Finally, Manole *et al.* (2015) point out to create areas of exposed ground where *Adenophora liliifolia* seeds could germinate (clearings), impede mowing and grazing, and remove invasive species as some *in situ* measures.

### III.4.3. *Ex situ* conservation

*In situ* conservation is a primary issue and an advantage over *ex situ* conservation methods. However, in some cases, *ex situ* methods are the only possibilities for the survival of species in habitat degradation or indirect destruction of the population (Kovács & Tillyné, 2019). Moreover, the commercial production of native species in an endangered situation can be accompanied by *ex situ* conservation (Karlović, 2009).

Approaches to *ex situ* conservation of genetic resources include different methods like seed storage, *in vitro* storage, DNA storage, pollen storage, field gene banks, and botanical gardens (Radha *et al.*, 2012; Malhotra *et al.*, 2019).

The first method, seed storage, is the collection of seed samples at one location and their transfer to a genebank for storage. The samples are usually dried to suitably low moisture content (i.e., equilibrium relative humidity (eRH) 15-25%) and then kept at sub-zero temperatures (e.g., -20°C) (Hawkes *et al.*, 2000; Popova *et al.*, 2015).

Seed storage requires relatively low maintenance, and specialized equipment is not needed when implemented on small scales. However, seed banking is not a viable option for some seeds with short lifespans or plants that do not produce heterozygous or any seeds as coconut (*Cocos nucifera* L.), cacao (*Theobroma cacao* L.), avocado (*Persea americana* Mill.), citrus (*Citrus* spp. L.), edible banana (*Musa* spp. L.) or garlic (*Allium sativum* L.). Species including yucca (*Yucca* sp. L.) and bamboo (*Bambuseae* Kunth ex Dumort) that have long life cycles and take years or decades to reproduce also fall into this category. Other species that produce orthodox seeds but require the conservation of particular gene combinations or genotypes, such as root and tuber crops, notably potato (*Solanum tuberosum* L.), cassava (*Manihot esculenta* Crantz), and several fruits and nut trees, are also included. These crops are propagated vegetatively, and each genotype needs to be maintained as a clone (Davies *et al.*, 2018; Nage *et al.*, 2019; Werden *et al.*, 2020; Panis *et al.*, 2020).

The second method, *in vitro* storage, is the collection and maintenance of explants (tissue samples) in a sterile, pathogen-free environment (Hawkes *et al.*, 2000). *In vitro* germplasm conservation focuses on controlling the normal growth of explants and slowing it by manipulating either the culture medium's constitution and/or storage conditions. Two ways of storing *in vitro* germplasm are slow growth or cryopreservation (Jaramillo & Baena, 2002). From these two ways of keeping, new cryopreservation techniques - stored at ultra-low temperatures in LN (Liquid Nitrogen) (-196°C) or its vapor phase (-150°C) - are successfully employed with all explant types, including cells suspensions and calluses, apices, and somatic and zygotic embryos of temperate and tropical species (Engelmann, 2004; Radha *et al.*, 2012).

Efficient cryopreservation protocols have been developed for many plant species showing a low risk of loss of the preserved samples. The significant disadvantages of this method are the requirement of an expensive program freezer and relatively long exposure of samples to subzero temperatures, which can be deleterious for cold-sensitive species (Popova *et al.*, 2015; Niino & Valle, 2015).

Cryopreservation enables long-term conservation of Critically Endangered species like *Rubus humulifolius* C.A.Mey. (Edesi *et al.*, 2020) or rare and Endangered Plant Species like *Cosmos atrosanguineus* (Hook.) Voss (Wilkinson *et al.*, 2003) and more.

The third method, Field Genebank, could be defined as collecting seed or living material from one location and its transfer and planting at a second site. Large numbers of accessions of a few species are usually conserved. The place for a field genebank should have a suitable climate and soil for the species and requires an adequate water supply with little or no threat of pests, diseases, bush fire, and vandalism (Hawkes *et al.*, 2000; Said & Ramanatha, 2001).

The fourth method, DNA / Pollen Storage, is the collection of DNA or pollen and storage in appropriate, usually refrigerated, conditions. It is necessary to mention that pollen storage does not conserve the whole plant genome in its haploid state; however, it can be an essential source of genetically diverse and heritable character traits in plant breeding programs that utilize artificial pollination (Hawkes *et al.*, 2000; Nadarajan *et al.*, 2018).

The fifth method, Botanic Garden/Arboretum, is defined as collecting seed or living material from one location and its transfer and maintenance at a second location as living plant collections of species in a garden or an arboretum. More than a third of plant species is represented in botanic gardens collections; moreover, small numbers of accessions of a wide range of species are usually conserved (Hawkes *et al.*, 2000). Living collections in botanical gardens and arboretum are significant for preserving critically endangered species or species extinct in the wild, which cannot be conserved through seed or tissue-banking protocols (Volis, 2017). Worldwide, there are over 3,731 botanic gardens, and they make the world's plant species diversity known to the public (BGCI, 2021; Chen & Sun, 2018).

Manole *et al.* (2015) mention that *ex situ* conservation measures for *A. liliifolia* should be taken to preserve species germplasm as seeds (in seed banks) or by cultivation.



### III.5. *In vitro* propagation

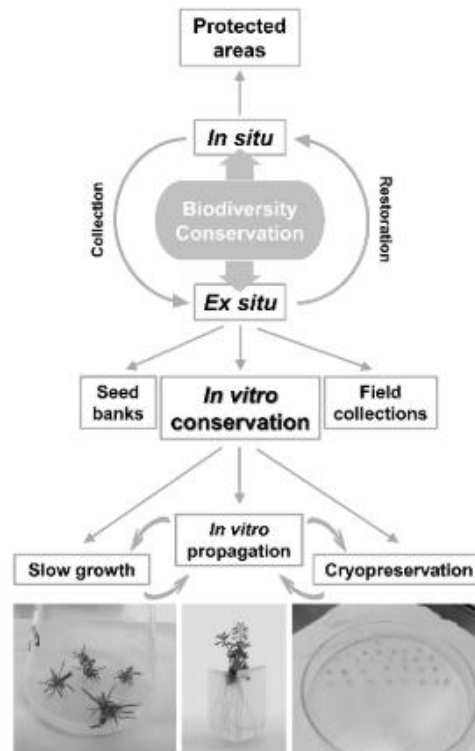
Micropropagation is the clonal propagation of plants in closed vessels under aseptic conditions. Inside the vessels, the plants are grown on culture media that contain nutrients and growth regulators. They are described as *in vitro*, which means 'in glass' (Roberts & Schum, 2003).

*In vitro* techniques have been successfully used to propagate a wide range of endangered taxon and are considered one of the most important *ex situ* conservation policies (Fay, 1992; Manal *et al.*, 2014). The *in vitro* techniques for conserving plant biodiversity include shooting apical or axillary-meristem-based micropropagation, somatic embryogenesis, cell culture technologies, and embryo rescue techniques, as well as a range of *in vitro* cold storage and cryopreservation protocols (Reed *et al.*, 2011; Chauhan, 2016).

The reasons for undertaking *in vitro* propagation of plants are varied (Park, 2021):

- a) Clonal integrity of the mother plant is maintained.
- b) Propagation through seed results in progeny that can vary in important traits.
- c) Some plants do not have viable seeds.
- d) Pathogen-free stock plants can be maintained.
- e) Material *in vitro* can be cryopreserved in germplasm banks.
- f) Endangered species can be regenerated in large numbers.
- g) Desirable parent stocks can be generated to produce hybrid seeds.
- h) Using cell culture techniques, it is possible to produce large numbers of identical plants rapidly.

Micropropagation or *in vitro* propagation of threatened plants has been a time-tested and effective method of *ex situ* conservation (Fig. 4.) (Reed *et al.*, 2011). Recent examples from around the world of *in vitro* propagation of threatened plants which try to establish protocols to save the species are the *ex situ* conservation of *Arnebia euchroma* (Royle) Johnston, a Himalayan medicinal herb that has become critically endangered because of indiscriminate harvesting (Chawla *et al.*, 2020; Chawla *et al.*, 2021).



**Figure 4.:** Schematic representation of different conservation strategies focuses on biotechnological-based techniques (Coelho *et al.*, 2020).

Likewise, in Saint Katherine Protectorate, one of the largest protected areas in Egypt, the establishment of a protocol for the endangered species *Silene schimperiana* Boiss. was necessary because of the decreasing of its population by anthropogenic activities, climate change, pollution, and exploitation of natural resources (Ghareb *et al.*, 2020; Omar & Elgamal, 2021).

Furthermore, the endemic plant *Magnolia sirindhorniae* Noot. & Chalermglin from Thailand has a high ornamental and commercial value. However, there is a decline in its habitat and difficulty reproducing by seeds because low percentage of fruit set. This species was classified as 'Endangered' on the IUCN Red List. The decrease in its population made scientists develop an efficient micropropagation protocol to preserve this species (Cui *et al.*, 2019; Global Tree Specialist Group, 2014).

In recent years (from 2015 to 2017), the Czech Republic organized a Project called "Action Plan for the Lilyleaf Ladybells (*Adenophora liliifolia*)" to preserve this species and stop the decrease in its population in Středočeský, Ústecký, Královehradecký regions. The project developed a methodology of *in vitro* cultivation. As a result, many new plants were obtained quickly (NCA CR, 2017).

Micropropagation has many benefits, such as a high multiplying rate, small stresses on the number of initial plants, a small quantity of space requirement, and multiplying of plants independently from the seasons of the year. In contrast, a significant disadvantage is the high demand cost (Maryam *et al.*, 2014).

### III.5.1. Medium

Plant tissue culture medium contains all the nutrients required for the normal growth and development of plants. It is mainly composed of macronutrients, micronutrients, vitamins, other organic components, plant growth regulators, carbon sources, and some gelling agents in a solid medium. Murashige and Skoog medium (MS) is most extensively used for the vegetative propagation of many plant species *in vitro*. The pH of the media is also important that affects both the growth of the plants and the activity of plant growth regulators. It is adjusted to the value between 5.4 - 5.8. Both the solid and liquid mediums can be used for culturing (Oseni *et al.*, 2018).

### III.5.2. Plant growth regulators and natural extracts

Plant growth regulators (PGRs) are natural hormones and synthetic hormone analogues. PGRs play an essential role in determining the development pathway of plant cells and tissues in a culture medium. The auxins, cytokinins, and gibberellins are the most used PGRs. The type and the concentration of hormones used depend mainly on the plant species, the tissue or organ cultured, and the experiment's objective. Auxins and cytokinins are the most widely used plant growth regulators in plant tissue culture, and their amount determined the type of culture established or regenerated. The high concentration of auxins generally favors root formation, whereas the high concentration of cytokinins promotes shoot regeneration. A balance of both auxin and cytokinin leads to the development of a mass of undifferentiated cells known as callus (Small & Degenhardt, 2018; Oseni *et al.*, 2018).

Some examples of commonly used PGRs are Auxins as Indole-3-acetic acid (IAA), 1-Naphthaleneacetic acid (NAA), or Indole-3-butyric acid (IBA). Cytokinins as 6-Benzylaminopurine (BAP), Kinetin (KIN). Miscellaneous PGRs as Gibberellic acid GA3 (Merck, 2021).

Another element as coconut water (coconut liquid endosperm), is traditionally used as a growth supplement in plant tissue culture/micropropagation. The wide applications of coconut water can be justified by its unique chemical composition of sugars, vitamins, minerals, amino acids, and phytohormones (Yong *et al.*, 2009). Moreover, the use of ethylene inhibitors like silver nitrate is found to be beneficial for shoot multiplication, rooting, and in plant tissue culture studies and bactericide action (Giridhar *et al.*, 2001; Kumar *et al.*, 2009).

### III.5.3. Acclimatization

The abnormal physiological and anatomical characteristics of micropropagated plantlets require that they should be gradually acclimatized to the environment of the greenhouse or field. Environmental factors, especially relative humidity, and irradiance, play a critical role in physiological and biochemical functions.

Throughout this period, substantial changes in those characteristics are necessary to achieve successful micropropagation (Osório *et al.*, 2013).

### III.6. Case studies

Sterile sowing and micropropagation techniques are essential to provide the opportunity to grow plant material under controlled conditions and preserve genetic diversity. Other methods like clonal propagation are less popular for protected species; however, in some cases, it is justified to use it to increase the number of individuals (Kovács & Tillyné, 2019).

Seed germination can be increased notably using *in vitro* methods in some species, where conventional techniques achieve low or no germination due to dormancy or specific requirements needed for germination (Fay, 1992). For example, to preserve and introduce new naturalized valuable to the floriculture markets, Seglie *et al.* (2012) investigated *in vitro* seed germination and seedling multiplication methods for *Campanula* species (*C. barbata* L., *C. latifolia* L., *C. rapunculoides* L., *C. spicata* L., and *C. trachelium* L.) from northern Italy. Under optimal conditions, seedlings produced shoots within three months, and the optimal medium for growing *Campanula* plants was full-strength MS enriched with 4 mg L<sup>-1</sup> BAP.

Considering other species cases, Cerabolini *et al.* (2004) studied seed germination dynamics of *Physoplexis comosa* (L.) Schur. and *Primula glaucescens* Moretti., endemic to calcareous grasslands of the Lombardy Prealps in northern Italy, to produce appropriate germination protocols for *ex situ* conservation. They demonstrated that *ex situ* propagation is possible from seeds; likewise, it maximizes genetic variability.

Considering another type of explant, Paunescu (2010) worked with single-node aerial stem segments from mature flowers of *Campanula polymorpha*, an endemic plant of the Carpathian Mountains. The best rate of shoot production (average 14.8 shoots/explant) was achieved after five weeks of culture on media supplemented with 0.1 mg L<sup>-1</sup> NAA and 1 mg L<sup>-1</sup> BAP. Furthermore, Chen *et al.* (2001) established a tissue culture protocol using stem internode explants of *Adenophora triphylla* (Thunb.) A. DC., an essential medicinal herb in Taiwan. Adventitious shoots formed a new crop of multiple shoots when subcultured on MS medium supplemented with 17.75 µM BAP. Shoots were rooted on 1/4-strength MS basal medium supplemented with 5.37 µM NAA. Plantlets were successfully propagated and replanted in the natural habitat; consequently, it prevents the depletion of the natural population.

## IV. MATERIALS AND METHODS

### IV.1. Materials

The research was conducted mainly in the Laboratory of Micropropagation, Department of Floriculture and Dendrology, Hungarian University of Agriculture and Life Sciences, Budapest, 2019-2021. The acclimatization experiment was carried out in the orchid greenhouse of Eötvös Loránd University (ELTE) Botanical Gardens, Budapest, 2021.

The experiment required seeds of *Adenophora liliifolia*; they were collected from two sources:

- a) Seeds of *Adenophora liliifolia* collected from randomly selected individuals of a natural population in Ócsa (GPS coordinates: 47°15'42" 19°15'35", alt.(m): 247) in September 2018. The population was small, consisting of approx. ten individuals scattered over the area. The habitat is a riparian mixed gallery forest (Prausová *et al.*, 2016).
- b) Seeds of *Adenophora liliifolia* (Lily-leafed Lady Bells) –Item No. AA112 from Jelitto® (n. d.)

Seeds were used as explants, but only for culture initiation and sterilized plantlets for further experiments. The plantlet that germinated *in vitro* were used as explants. Sixty individuals from *in vitro* seeds were obtained.

#### IV.1.1. Culture media and components used during *in vitro* propagation

- a) Silver nitrate ( $\text{AgNO}_3$ ) - the growing medium was  $\frac{1}{2}$  MS, MS/2 macronutrients, MS micronutrients, MS vitamins + 100 mg m-Inositol, 25 mg Fe-EDTA, 20 g sugar, 7 g agar, pH: 5.8. The following doses of silver nitrate were incorporated into the experiment:

- Control (0 mg L<sup>-1</sup>)
- 5 mg L<sup>-1</sup>
- 10 mg L<sup>-1</sup>
- 20 mg L<sup>-1</sup>

- b) 1-Naphthaleneacetic acid (NAA) - the growing medium was  $\frac{1}{2}$  MS. The following doses of NAA were used for the experiment:

- Control (0 mg L<sup>-1</sup>)
- 0.1 mg L<sup>-1</sup>
- 0.5 mg L<sup>-1</sup>
- 1 mg L<sup>-1</sup>

c) Coconut water (CW) - the growing medium was  $\frac{1}{2}$  MS + MS micro + MS vitamin + 50 mg Fe-EDTA + 20 g sugar + 6 g agar. The following doses of CW were used for the experiment:

- Control (0 ml L<sup>-1</sup>)
- 25ml L<sup>-1</sup>
- 50ml L<sup>-1</sup>
- 100ml L<sup>-1</sup>
- 200ml L<sup>-1</sup>

d) pH gradient - the growing medium was  $\frac{1}{2}$  MS. The adjustment of pH was made by adding 1 N KOH and/or 1N HCl. The following pH adjustments were used for the experiment:

- pH 5.6-5.8
- pH 6.8-7
- pH 7.8-8

#### **IV.1.2. Chlorophyll and Carotenoids content**

After finishing the experiments of coconut water treatment and pH gradients, the explants were stored in a climate chamber at -37°C for five months. These explants were used for Chlorophyll and Carotenoids content determination.

#### **IV.1.3. Substrate mixtures used for acclimatization**

Four substrates were elaborated in a proportion of 1:1:1 (v/v):

- S<sub>1</sub> (small pieces of pine bark, wetted sphagnum moss, perlite)
- S<sub>2</sub> (small pieces of pine bark, sphagnum moss, small pieces of zeolite stone)
- S<sub>3</sub> (coconut fiber, sphagnum moss, perlite)
- S<sub>4</sub> (coconut fiber, sphagnum moss, zeolite)

These substrates were settled in two systems:

- CA (covered with agronet)
- UN (uncovered)

## IV.2. Methods

### IV.2.1. Experiments with different medium components

Plantlet's manipulations were carried out in the laminar airflow box (BA-900, manufacturer: Debreceni Finommechanikai Vállalat). Plantlets were cultured on top-illuminated growth shelves at  $22 \pm 2^\circ\text{C}$  temperature, 16/8 hours photoperiod, and 3000 lx luminance during multiplication.

The following parameters were measured:

- Plant height: the length of the most extensive shoot in one conglomerate, mm.
- No. of shoots: the number of live shoots produced by the initial plantlet, pcs.
- No. of leaves: the number of alive leaves produced by the initial plantlet, pcs.
- No. of roots: the number of roots produced per individual plant, pcs.
- Root length: the length of the longest root per individual plant, mm.
- Leaf length: the length of each leaf from the pointy part at the point where the leaf joins the stalk at the other end, mm.
- Leaf width: the most extended area of two points on the blade edge perpendicular to the leaf length axis, mm.

### IV.2.2. Chlorophyll and Carotenoids content

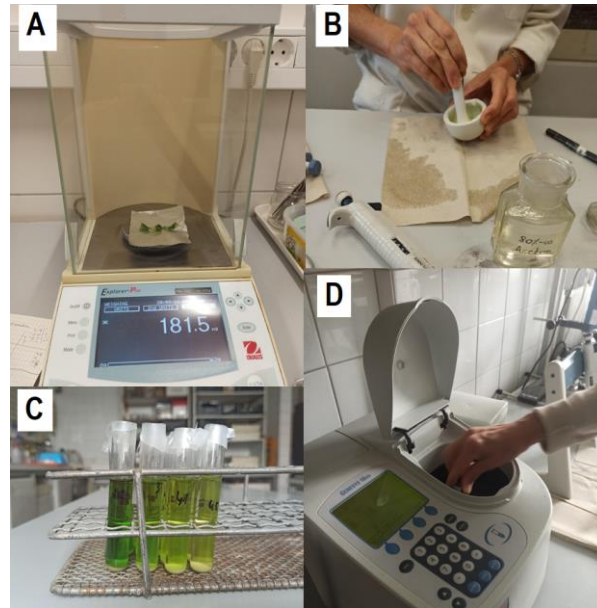
The fresh weight (whole shoot clumps, consisting of stems and leaves) of four samples for each treatment were used for the determination of chlorophyll and carotenoids content of the coconut water treatment ( $152.1 \pm 22.54$  mg per sample) and pH gradients ( $163.7 \pm 15.64$  mg per sample) according to Arnon (1949).

Samples were homogenized in 5 ml for pH gradients and 10 ml for Coconut water, 80% (v/v) acetone was used as a solvent in a precooled mortar with a pestle, and a small amount of quartz sand to help the homogenization. The homogenized suspension was left for one night to settle the tissue remains and quartz sand. Light absorbance of the solution was measured in Genesys 10vis type spectrophotometer at three different wavelengths ( $A_{480}$  nm,  $A_{644}$  nm,  $A_{663}$  nm) (Fig.5.). Later, the chlorophyll and carotenoids content were calculated in Microsoft Office Excel using the following formula:

$$\text{Chlorophyll a + b } (\mu\text{g/mg}) = \frac{(20.2 \cdot A_{644} + 8.02 \cdot A_{663}) \cdot X \text{ ml}}{\text{weight}}$$

$$\text{Carotenoids } (\mu\text{g/mg}) = \frac{5.01 \cdot A_{480} \cdot X \text{ ml}}{\text{weight}}$$

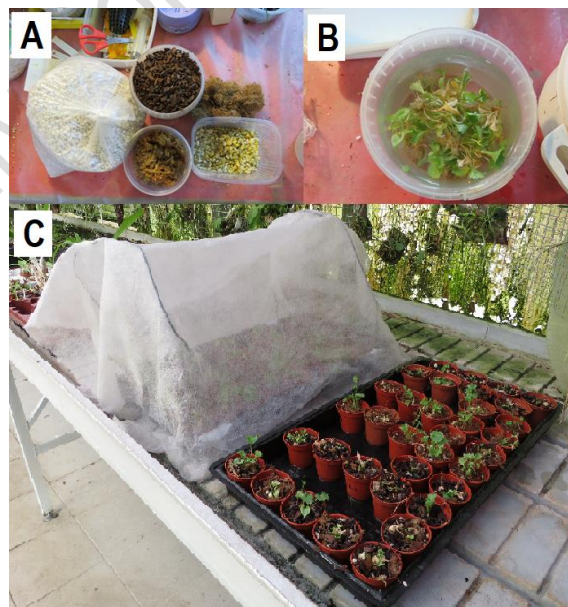
X=10 ml for Coconut water or 5 ml for pH gradients



**Figure 5.:** Chlorophyll a + b and Carotenoids experiment: A. weighing the samples, B. grinding the leaves C. homogenized suspension, and D. measuring in the spectrophotometer (photos by the author).

#### IV.2.3. Method of acclimatization

First, plants were removed from the test tube, then rinsed in tap water for 10 minutes to remove the MS medium from the roots surface. After, *A. liliifolia* were planted in a 6 cm diameter pot in the above-described substrates (S<sub>1</sub>, S<sub>2</sub>, S<sub>3</sub>, S<sub>4</sub>). Half of the plants were covered with agronet, others not. All the plants were irrigated with 6-7 ml of 2 g/l Dithane M 45 containing rainwater solution (Fig.6.).



**Figure 6.:** *A. liliifolia* acclimatization experiment: A. substrates, B. *A. liliifolia* plants removed from *in vitro* culture, and C. cover and uncover trials (photos by Tillyné Mándy Andrea).



The plants were located in a glasshouse of ELTE Botanical Garden under a temperature of 24°C, 80% air humidity for one month.

The following parameters were measured:

- Plant height: point on the stem where roots start to grow to the highest fully expanded leaf, cm.
- Plant diameter: the horizontal size of the plant, cm.
- No. of leaves: the total number of alive leaves, pcs.
- Leaf length: the length of each leaf from the pointy part at the point where the leaf joins the stalk at the other end, cm.
- Leaf width: the most extended area of two points on the blade edge perpendicular to the leaf length axis, cm.

#### IV.2.4. Statistical analysis

Based on the information collected during the experimental work, databases were created in Microsoft® Excel® for Microsoft 365 MSO (Version 2109).

The experiments were established in a completely randomized design. Data was examined by applying the analysis of variance one-way MANOVA or two-way MANOVA using IBM® SPSS® Statistics version 27.0.1 software to detect significant differences between means.

The normality of the residuals was tested according to their Skewness and Kurtosis (D'Agostino *et al.*, 1990; Tabachnick & Fidell, 2013;  $p > 0.05$ ) or Shapiro - Wilk Test (Shapiro & Wilk, 1965). In some cases, when the presence of multivariate outlier, lambda of Box-Cox transformation (Box & Cox, 1964) was used. The cases will be mentioned. Homogeneity of variances was tested by Levene's method (Levene, 1960).

Means were compared using Tukey HSD (Dunn, 1961) or Games-Howell post hoc test (Field, 2013) depending on whether the homogeneity of variances was accepted or violated, at a 5% probability level.

##### a) Silver Nitrate ( $\text{AgNO}_3$ )

To compare the effect of the treatments (control, 5 mg L<sup>-1</sup>, 10 mg L<sup>-1</sup>, 20 mg L<sup>-1</sup>) on dependent variables Shoot length [mm], No. of shoots, and No. of roots for *A. liliifolia*; one-way MANOVA was applied. Every treatment was  $n = 36$  ( $N = 144$ ).

As assessed by box plot methods, the original data showed two univariate outliers in No. of roots. Also, Mahalanobis Distance Test identified one multivariate outlier.

After Box-Cox data transformation of the variable No. of roots with Lambda value ( $\lambda$ ) = 0, and  $\log(Y)^{**} = -117.292$ ; box plot and Mahalanobis Distance Test did not detect any univariate and multivariate outliers, respectively.

The normality of the residuals (obtained from the transformed dataset) was accepted by the absolute values of their Skewness and Kurtosis. These values were smaller than 1 in all variables, except for the Skewness value in No. of roots which showed a bit above 1 but less than 2. However, normality was not seriously violated.

Homogeneity of variances was violated in the case of Shoot length: Levene's Test showed  $F(3,140) = 5.525$ ,  $p < 0.05$ . Homogeneity of variances was accepted for the variables No. of shoots ( $F(3,140) = 1.232$ ,  $p = 0.301$ ) and No. of roots ( $F(3,140) = 0.337$ ,  $p = 0.798$ ).

#### **b) 1-Naphthaleneacetic acid (NAA)**

To compare the effect of the treatments (control, 0.1 mg L<sup>-1</sup>, 0.5 mg L<sup>-1</sup>, 1 mg L<sup>-1</sup>) on dependent variables Plant height [mm], Leaf length [mm], Leaf width [mm], Root length [mm], No. of roots, No. of shoots for *A. liliifolia*; one-way MANOVA was applied. Every treatment was  $n = 39$ ,  $n = 40$ ,  $n = 44$ ,  $n = 45$ , respectively ( $N = 168$ ).

The original data showed 11 univariate outliers in Plant height (5), Leaf length (1), No of roots (4), and Root length (1), as assessed by box plot methods. Also, Mahalanobis Distance Test identified two multivariate outliers.

After Box-Cox data transformation of the variables Plant height ( $Y_1 = Y = -477.732$ ), Leaf length ( $Y_1 = Y = -290.301$ ), Root length ( $Y_1 = Y = -351.46$ ) with Lambda values ( $\lambda$ ) = 1, and No. of roots ( $Y_1 = Y = -241.426$ ) with Lambda value ( $\lambda$ ) = 0.5; the Mahalanobis Distance Test did not detect multivariate outliers.

The normality of the residuals (obtained from the transformed dataset) was checked by the absolute values of their Skewness and Kurtosis. These values were smaller than 1 in all variables.

Homogeneity of variances was violated in case of Root length: Levene's Test showed  $F(3;164) = 4.186$ ,  $p < 0,05$ . However, Homogeneity of variances was accepted for the variables Plant height ( $F(3,164) = 0.816$ ,  $p = 0.487$ ), Leaf length ( $F(3,164) = 0.365$ ,  $p = 0.779$ ), Leaf width ( $F(3,164) = 0.384$ ,  $p = 0.765$ ), No. of roots ( $F(3,164) = 1.372$ ,  $p = 0.253$ ), and No. of shoots ( $F(3,164) = 1.360$ ,  $p = 0.257$ ).

### c) Coconut water treatment

To compare the effect of the treatments (control, 25 ml L<sup>-1</sup>, 50 ml L<sup>-1</sup>, 100 ml L<sup>-1</sup>, 200 ml L<sup>-1</sup>) on dependent variables Plant height [mm], Root length [mm], No. of roots, No. of shoots for *A. liliifolia*; one-way MANOVA was applied. Every treatment was n = 21 (N = 105).

The original data identified seven univariate outliers in the variables Plant height (1), Root length (2), No. of roots (1), and No. of shoots (3), as assessed by box plot methods. Using Mahalanobis Distance Test, one multivariate outlier was identified.

Box-Cox data transformation of the variables Plant height ( $Y_{-0.5} = 1/(\sqrt{Y}) = -182.241$ ) with Lambda value ( $\lambda$ ) = -0.5, No. of shoots ( $\log(Y)^{**} = -38.246$ ) with Lambda value ( $\lambda$ ) = 0, and Root length ( $Y_{0.5} = \sqrt{Y} = -208.01$ ), and No. of roots ( $Y_{0.5} = \sqrt{Y} = -160.999$ ) with Lambda values ( $\lambda$ ) = 0.5 were necessary.

After data transformation, Mahalanobis Distance Test did not detect multivariate outliers. Moreover, the normality of the residuals of the variables (obtained from the transformed dataset) was accepted by the absolute values of Skewness and Kurtosis. These values were smaller than 1 in all variables. However, Kurtosis of No. of shoots was a bit higher than 1 but below 2. Normality was not seriously violated.

Homogeneity of variances was violated in case of No. of roots: Levene's Test showed  $F(4,100) = 2.946$ ,  $p < 0.05$ . However, Homogeneity of variances was accepted for the variables Plant height ( $F(4,100) = 1.137$ ,  $p = 0.343$ ), Root length ( $F(4,100) = 1.237$ ,  $p = 0.300$ ), and No. of shoots ( $F(4,100) = 0.411$ ,  $p = 0.800$ ).

### d) pH level treatment

To compare the effect of the treatments (pH 5.6 - 5.8, pH 6.8 - 7, and pH 7.8 - 8) on dependent variables Plant height [mm], Leaf length [mm], Leaf width [mm], No. of roots, No. of shoots, and No. of leaves for *A. liliifolia*; one-way MANOVA was applied. Every treatment was n = 24 (N = 72).

Three univariate outliers were identified in the variables Plant height, No. of roots, and No. of leaves, as assessed by box plot methods. Using Mahalanobis Distance Test, no multivariate outliers were identified.

The normality of the residuals of the variables was approved by the absolute values of Skewness and Kurtosis. These values were smaller than 1 in all variables. However, Kurtosis in the variable No. of leaves was a bit above 1 but below 2. The normality was not seriously violated.

Homogeneity of variances was violated in case of Plant height and No. of shoots: Levene's Test showed  $F(2,69) = 3.18$ ,  $p < 0.05$ , and  $F(2,69) = 3.561$ ,  $p < 0.05$ , respectively. However, Homogeneity of variances was accepted for the variables Leaf length ( $F(2,69) = 1.800$ ,  $p = 0.173$ ), Leaf width ( $F(2,69) = 1.724$ ,  $p = 0.186$ ), No. of roots ( $F(2,69) = 0.467$ ,  $p = 0.629$ ), and No. of leaves ( $F(2,69) = 0.590$ ,  $p = 0.557$ ).

#### **e) Chlorophyll a + b and Carotenoids of coconut water treatment**

To compare the effect of the treatments (control, 50 ml L<sup>-1</sup>, 100 ml L<sup>-1</sup>, and 200 ml L<sup>-1</sup>) on dependent variables Chlorophyll a + b [ $\mu\text{g}/\text{mg}$ ] and Carotenoids [ $\mu\text{g}/\text{mg}$ ] for *A. liliifolia*; one-way MANOVA was applied. Every treatment was  $n = 4$  ( $N = 16$ ).

As assessed by box plot methods, there were no univariate extreme outliers in the Chlorophyll a + b and Carotenoids variables. Using Mahalanobis Distance Test, no multivariate outliers were identified.

The normality of the residuals of the two variables Chlorophyll a + b and Carotenoid of *Adenophora liliifolia* that followed four different doses of coconut water treatment was approved by the absolute values Skewness and Kurtosis. Skewness values were a bit above 1 but below 2, and Kurtosis values were smaller than 1. The normality was not seriously violated.

Homogeneity of variances was accepted by Levene's test. Chlorophyll a + b:  $F(3,12) = 3.572$ ,  $p = 0.05$ , and Carotenoids:  $F(3,12) = 2.817$ ,  $p = 0.08$ .

#### **f) Chlorophyll a + b and Carotenoids of pH level treatment**

To compare the effect of the treatments (pH 5.6 - 5.8, pH 6.8 - 7, and pH 7.8 - 8) on dependent variables Chlorophyll a + b [ $\mu\text{g}/\text{mg}$ ] and Carotenoids [ $\mu\text{g}/\text{mg}$ ] for *A. liliifolia*; one-way MANOVA was applied. Every treatment was  $n = 4$  ( $N = 12$ ).

There were no univariate extreme outliers in the Chlorophyll a + b and Carotenoids variables, as assessed by box plot methods. Using Mahalanobis Distance Test, no multivariate outliers were identified.

Normality of residuals of the two variables Chlorophyll a + b and Carotenoid of *Adenophora liliifolia* that followed three different pH level treatment was accepted by Shapiro - Wilk Test (Chlorophyll a + b:  $W = 0.95$ ,  $p = 0.63$ ; Carotenoid:  $W = 0.92$ ,  $p = 0.29$ ).

Homogeneity of variances was violated in the case of Carotenoids: Levene's Test showed  $F(2,9) = 11.267$ ,  $p < 0.01$ . However, Homogeneity of variances was accepted for Chlorophyll a + b and showed  $F(2,9) = 3.701$ ,  $p = 0.07$ .

### g) Acclimatization

To compare the effect of type of substrates ( $S_1, S_2, S_3, S_4$ ) and type of cover (CA, UC) on dependent variables Plant height [cm], Plant diameter [cm], No. of leaves, Leaf length [cm], Leaf width [cm] for *A. liliifolia*; two-way MANOVA was applied. There were  $4 \times 2 = 8$  treatments with six replicates per treatment ( $N = 48$ ).

There were no univariate outliers in the variables, as assessed by box plot methods. Using Mahalanobis Distance Test, no multivariate outliers were identified.

The normality of the residuals of all the variables was accepted by the absolute values of Skewness and Kurtosis. These values were smaller than 1. Kurtosis in Leaf width was a bit above 1; however, normality was not seriously violated.

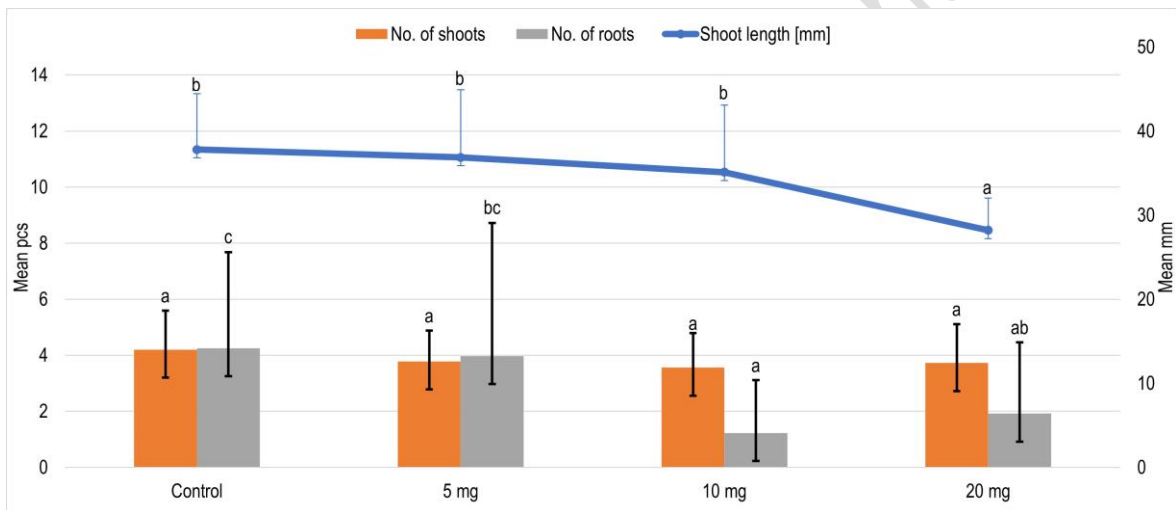
Homogeneity of variances was accepted for Plant height ( $F(7,40) = 1.683, p = 0.141$ ), and No. of leaves ( $F(7,40) = 1.566, p = 0.174$ ) by Levene's test. However, the variables Plant diameter ( $F(7,40) = 4.730, p < 0.001$ ), Leaf length ( $F(7,40) = 12.378, p < 0.001$ ), and Leaf width ( $F(7,40) = 5.660, p < 0.001$ ) were rejected by the same test.

## V. RESULTS

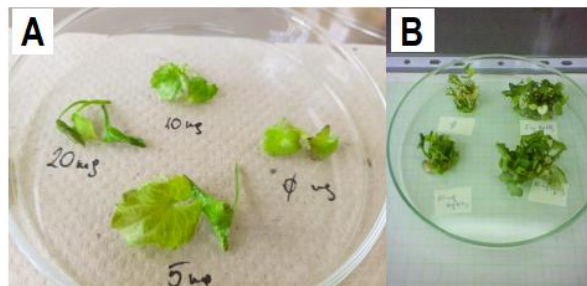
### V.1. Experiments with different medium components

#### V.1.1. Silver nitrate ( $\text{AgNO}_3$ )

During the comparison of the effect of  $\text{AgNO}_3$  treatments (control, 5 mg L<sup>-1</sup>, 10 mg L<sup>-1</sup>, and 20 mg L<sup>-1</sup>) on *Adenophora liliifolia*, one-way MANOVA proved statistically significant with Pillai's Trace = 0.371,  $F(9,420) = 6.582$ ,  $p < 0.001$ . The follow-up analysis revealed significant differences in the variables: Shoot length ( $F(3,140) = 14.528$ ,  $p < 0.001$ ) and No. of roots ( $F(3,140) = 8.687$ ,  $p < 0.001$ ). However, No. of shoots did not show significant difference ( $F(3,140) = 1.616$ ,  $p = 0.188$ ). A Games-Howell post hoc test was performed (Fig. 7 and 8.).



**Figure 7.:** Mean and standard deviation of Shoot length [mm], No. of shoots, and No. of roots of *A. liliifolia* that followed four different concentrations of  $\text{AgNO}_3$  (Control, 5 mg L<sup>-1</sup>, 10 mg L<sup>-1</sup>, 20 mg L<sup>-1</sup>). Different letters are for significantly different groups (Games-Howell  $p < 0.05$ ).



**Figure 8.:** Effect of different concentrations of  $\text{AgNO}_3$  on plant development (photo by Kovács Zsófia).

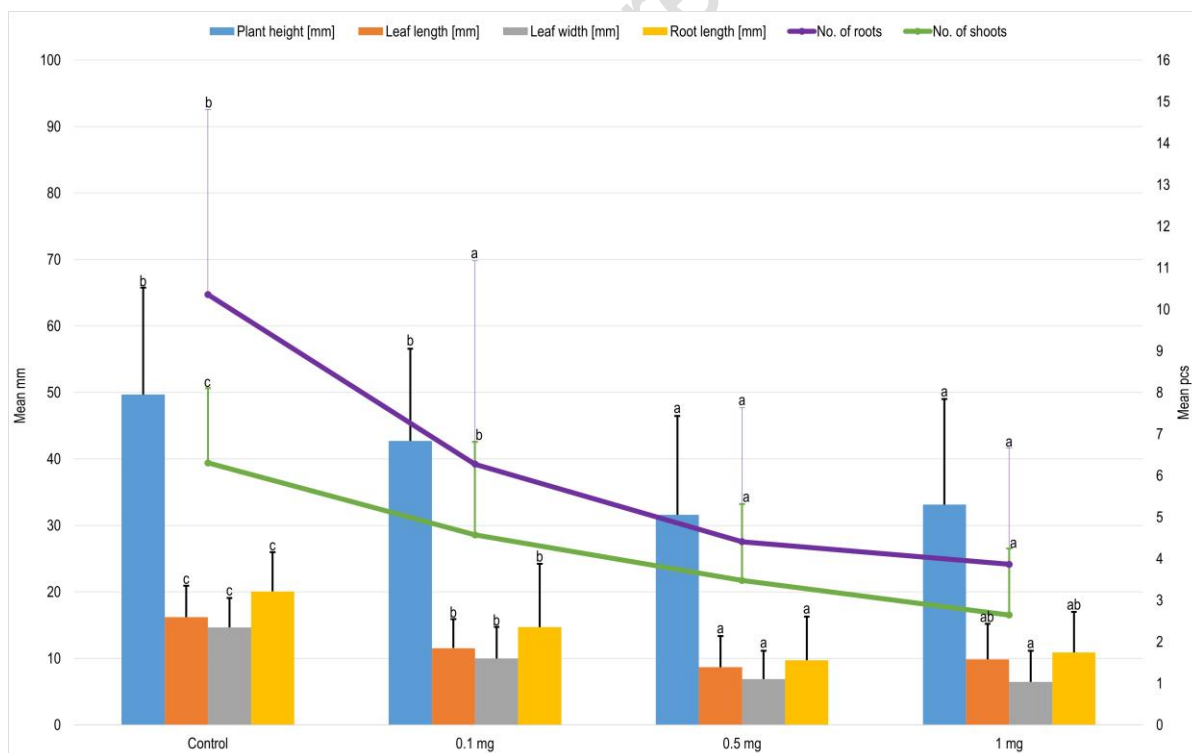
The experiment showed that a higher dose of  $\text{AgNO}_3$  negatively affects the number of roots generated in *A. liliifolia*; nevertheless, the variable No. of shoots was not affected for the concentration of

AgNO<sub>3</sub> in the *in vitro* medium. In the control medium, plants produced thin leaves, and most of them were vitrified.

Plants of reduced size, strongly reflective leaves developed on 20 mg L<sup>-1</sup> medium, and significant vitrification was observed. The medium containing 5 and 10 mg L<sup>-1</sup> had large, well-developed shoots and leaves, and minimal vitrification. The control treatment was statistically similar to 5 and 10 mg L<sup>-1</sup> in the variable shoot length.

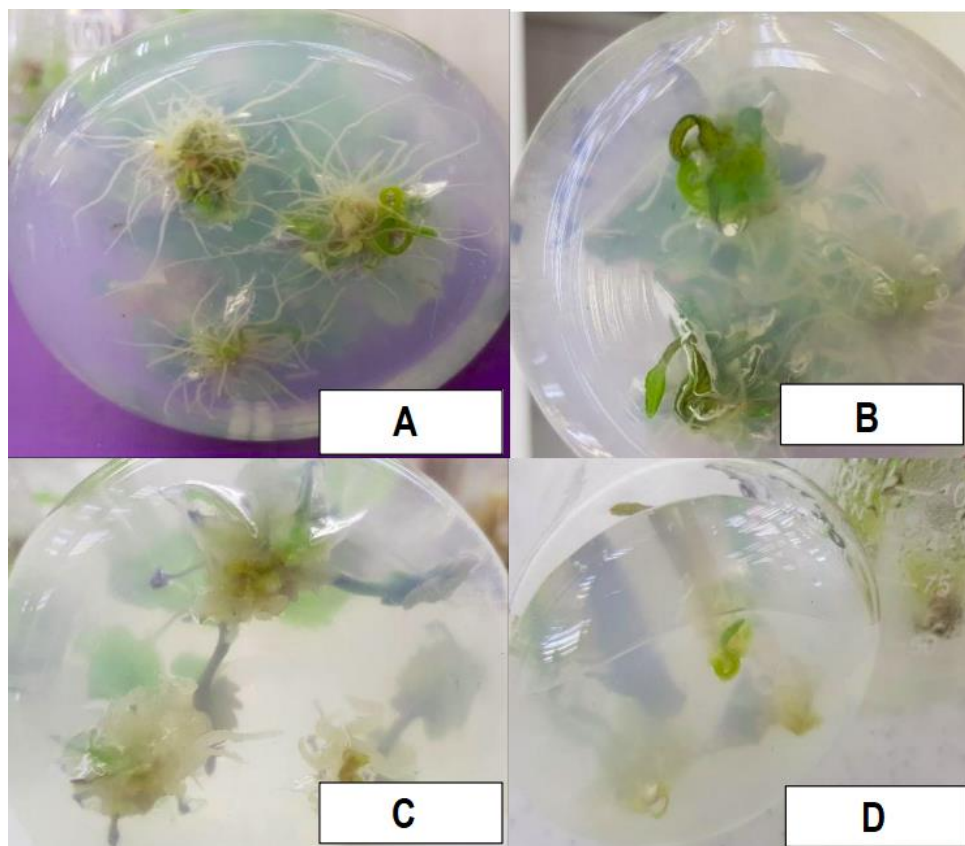
### V.1.2. 1-Naphthaleneacetic acid (NAA)

During the comparison of the effect of NAA treatments (Control, 0.1 mg L<sup>-1</sup>, 0.5 mg L<sup>-1</sup>, and 1 mg L<sup>-1</sup>) on *Adenophora liliifolia*, one-way MANOVA resulted statistically significant with Pillai's Trace = 0.591, F (18,483) = 6.584, p < 0.001. The follow-up analysis revealed significant differences in all the variables: Plant height (F (3,164) = 13.674, p < 0.001), Leaf length (F (3,164) = 19.240, p < 0.001), Leaf width (F (3,164) = 28.489, p < 0.001), Root length (F (3,164) = 15.970, p < 0.001), No. of roots (F (3,164) = 18.037, p < 0.001), and No. of shoots (F (3,164) = 29.513, p < 0.001). Games-Howell's post hoc test was performed (Fig. 9.).



**Figure 9.:** Mean and standard deviation of Plant height [mm], Leaf length [mm], Leaf width [mm], Root length [mm], No. of roots, and No. of shoots of *A. liliifolia* that followed four different concentrations of NAA (Control, 0.1 mg L<sup>-1</sup>, 0.5 mg L<sup>-1</sup>, 1 mg L<sup>-1</sup>). Different letters are for significantly different groups (Games-Howell post-hoc test, p < 0.05).

The control treatment showed a positive effect in all the variables and showed the best results in Plant height [mm], Leaf length [mm], Leaf width [mm], Root length [mm], No. of roots, and No. of shoots (Fig. 10.). Opposite to this, the doses 0.5 - 1 mg L<sup>-1</sup> performed the lowest effect in all the variables. The dose of 0.1 mg L<sup>-1</sup> had a similar statistical value to the control in the variable Plant height.

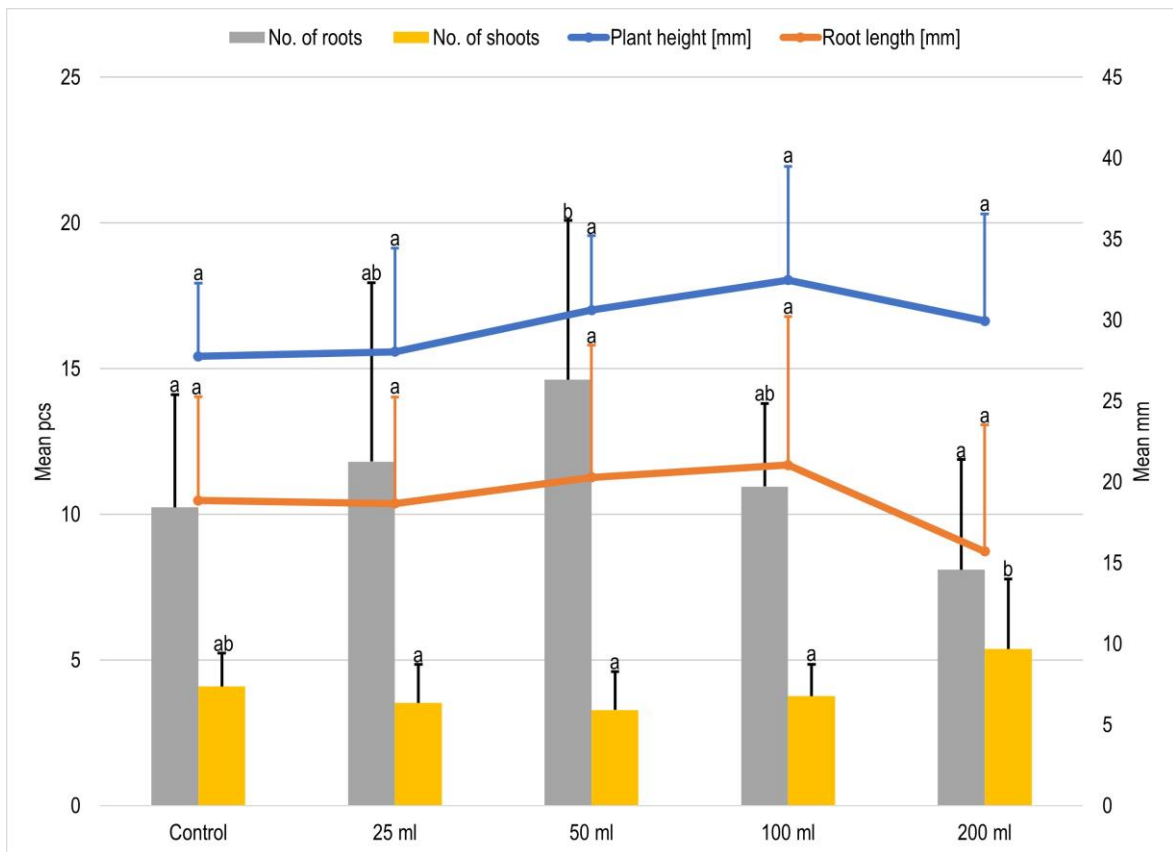


**Figure 10.:** Effect of NAA on root formation: A. Control, B. 0.1 mg L<sup>-1</sup>, C. 0.5 mg L<sup>-1</sup>, and D. 1 mg L<sup>-1</sup> (photos by Kovács Zsófia).

### V.1.3. Coconut water (CW)

During the comparison of the effect of CW treatments (Control, 25 ml L<sup>-1</sup>, 50 ml L<sup>-1</sup>, 100 ml L<sup>-1</sup>, 200 ml L<sup>-1</sup>) on *Adenophora liliifolia*, one-way MANOVA resulted statistically significant with Pillai's Trace = 0.536, F (16,400) = 3.865, p < 0.001. The follow-up analysis revealed that the differences were significant in the variables No. of shoots (F (4,100) = 6.302, p < 0.001) and No. of roots (F (4,100) = 5.844, p < 0.001). However, the variables Plant height (F (4,100) = 2.343, p = 0.06), and Root length (F (4,100) = 1.986, p = 0.102) did not show significant differences. Games-Howell's post hoc test was performed (Fig. 11.).





**Figure 11.:** Mean and standard deviation of Plant height [mm], Root length [mm], No. of roots, and No. of shoots of *A. liliifolia* that followed five different concentrations of CW (Control, 25 ml L<sup>-1</sup>, 50 ml L<sup>-1</sup>, 100 ml L<sup>-1</sup>, 200 ml L<sup>-1</sup>). Different letters are for significantly different groups (Games-Howell post-hoc test,  $p < 0.05$ ).

Doses in the range from 25 to 100 ml L<sup>-1</sup> positively affected the production of the number of roots but decreased the effect on the numbers of shoots. In contrast, a dose of 0 or 200 ml L<sup>-1</sup> individuals produced more shoots and less roots.

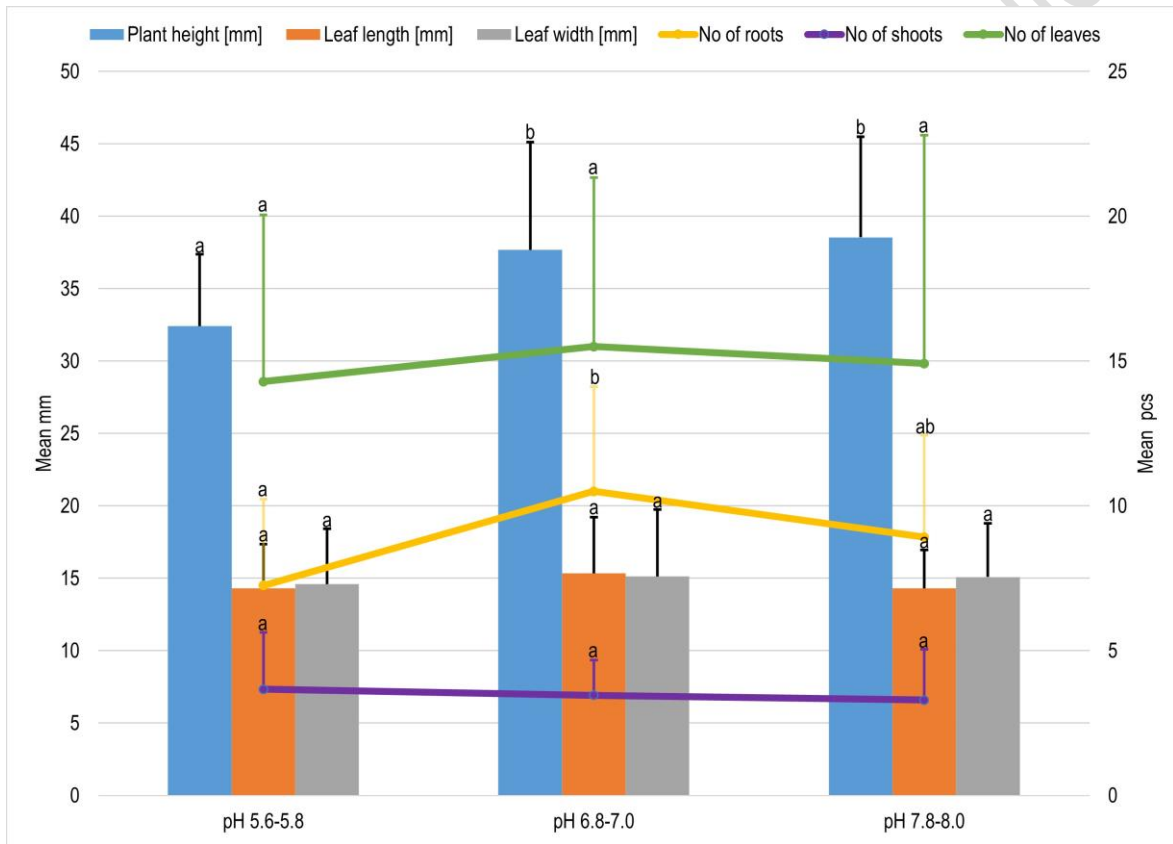
In terms of number of shoots, 200 ml L<sup>-1</sup> of CW medium was the most favorable, while the rooting rate was restrained. In terms of root number, 50 ml L<sup>-1</sup> of CW containing medium was ideal, combined with more restrained shoot formation (Fig. 12.).



**Figure 12.:** Well-developed plants on CW medium (photo by Kovács Zsófia).

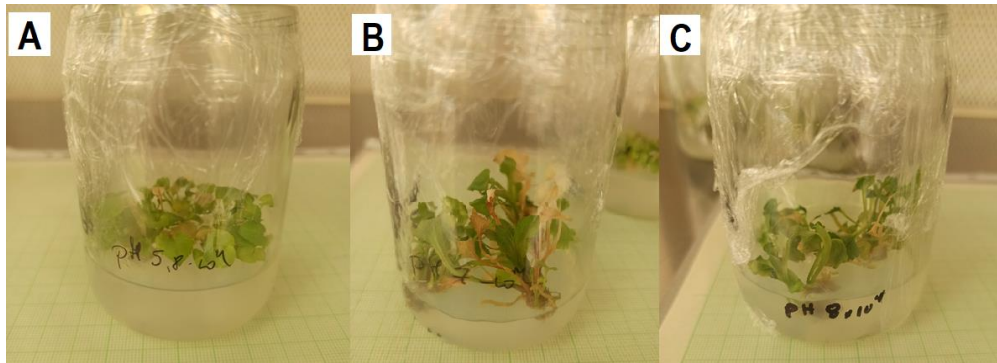
### V.1.4. pH optimization

During the comparison of the effect of pH treatments (pH 5.6 - 5.8, pH 6.8 - 7 and pH 7.8 - 8) on *Adenophora liliifolia*, one-way MANOVA resulted statistically significant with Wilk's  $\lambda = 0.633$ ,  $F(12,128) = 2.739$ ,  $p < 0.01$ . However, the follow-up analysis revealed that the differences were significant only in case of the variables Plant height ( $F(2,69) = 6.158$ ,  $p < 0.05$ ) and No. of roots ( $F(2,69) = 5.534$ ,  $p < 0.05$ ). The variables Leaf length ( $F(2,69) = 0.828$ ,  $p = 0.441$ ), Leaf width ( $F(2,69) = 0.131$ ,  $p = 0.877$ ), No. of shoots ( $F(2,69) = 0.301$ ,  $p = 0.741$ ), and No. of leaves ( $F(2,69) = 0.204$ ,  $p = 0.816$ ) did not show significant differences. Games-Howell's post hoc test was performed (Fig. 13.).



**Figure 13.:** Mean and standard deviation of Plant height [mm], Leaf length [mm], Leaf width [mm], No. of roots, No. of shoots, and No. of leaves of *A. liliifolia* that followed three different pH level (pH 5.6 - 5.8, pH 6.8 - 7, and pH 7.8 - 8). Different letters are for significantly different groups (Games-Howell post-hoc test,  $p < 0.05$ ).

The treatments pH 6.8 - 7 and pH 7.8 - 8 showed a statistically superior value in Plant height [mm] and No. of roots compared to the pH 5.6 - 5.8 treatment (Fig. 14.).

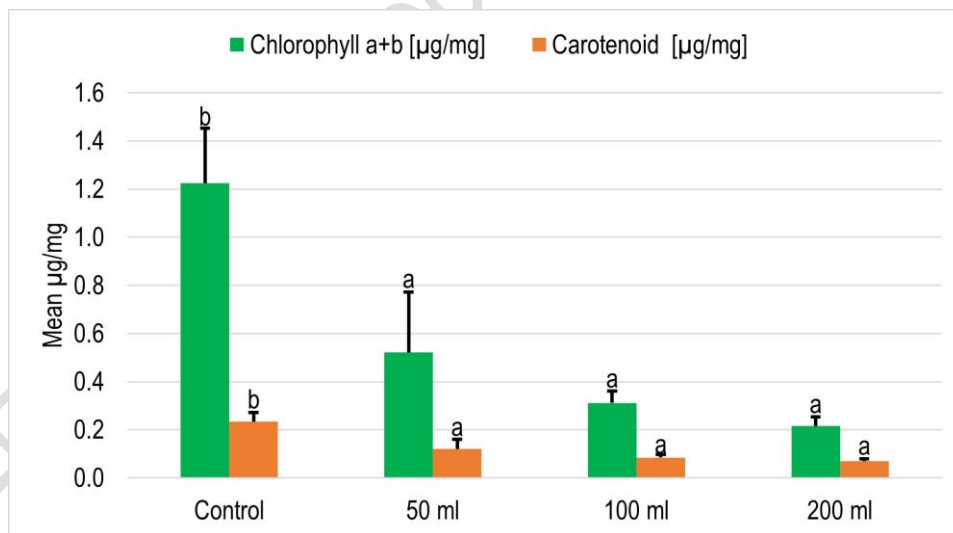


**Figure 14.:** pH optimization: A. pH 5.6 - 5.8, B. pH 6.8 - 7, and C. pH 7.8 - 8 (photos by the author).

## V.2. Chlorophyll and Carotenoids content

### V.2.1. Coconut water treatment

Chlorophylls and carotenoids are the main pigments in plants (Braniša *et al.*, 2016). During the comparison of the effect of CW treatments (control, 50 ml L<sup>-1</sup>, 100 ml L<sup>-1</sup>, and 200 ml L<sup>-1</sup>) on *Adenophora liliifolia* Chlorophyll a + b and Carotenoid content, one-way MANOVA resulted statistically significant with Wilk's  $\lambda = 0.123$ ,  $F(6,22) = 6.780$ ,  $p < 0.001$ . The follow-up analysis revealed there were significant differences in Chlorophyll a + b ( $F(3,12) = 27.817$ ,  $p < 0.001$ ) and Carotenoids ( $F(3,12) = 26.220$ ,  $p < 0.001$ ). Then, Tukey's post-hoc test was applied (Fig. 15).

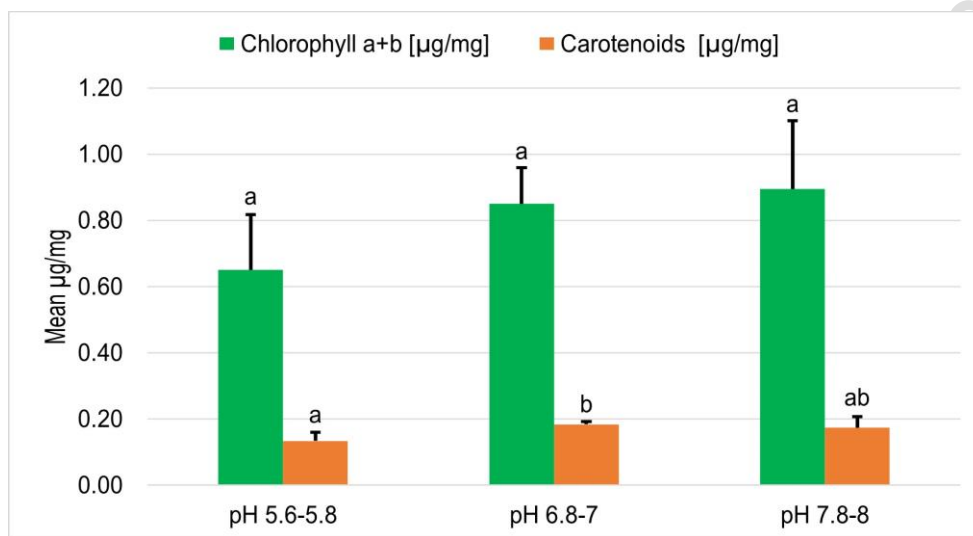


**Figure 15.:** Mean and standard deviation of Chlorophyll a + b [µg/mg] and Carotenoids [µg/mg] of *A. liliifolia* that followed four different concentrations of Coconut water (Control, 50 ml L<sup>-1</sup>, 100 ml L<sup>-1</sup>, 200 ml L<sup>-1</sup>). Different letters are for significantly different groups (Tukey HSD test  $p < 0.05$ ).

The control treatment showed a statistically significant value in Chlorophyll a + b and Carotenoid content than the doses of 50 ml L<sup>-1</sup>, 100 ml L<sup>-1</sup>, and 200 ml L<sup>-1</sup>.

### V.2.2. pH treatment

During the comparison of the effect of pH treatment (pH 5.6 - 5.8, pH 6.8 - 7 and pH 7.8 - 8) on *Adenophora liliifolia* Chlorophyll a + b and Carotenoid content, one-way MANOVA resulted statistically significant with Wilk's  $\lambda = 0.176$ ,  $F(4,16) = 5.528$ ,  $p < 0.01$ . The follow-up analysis revealed that the differences were significant only in the case of Carotenoid ( $F(2,9) = 4.364$ ,  $p < 0.05$ ). However, Chlorophyll a + b did not show significant differences ( $F(2,9) = 2.480$ ,  $p = 0.139$ ). Games-Howell's post hoc test was performed (Fig. 16).

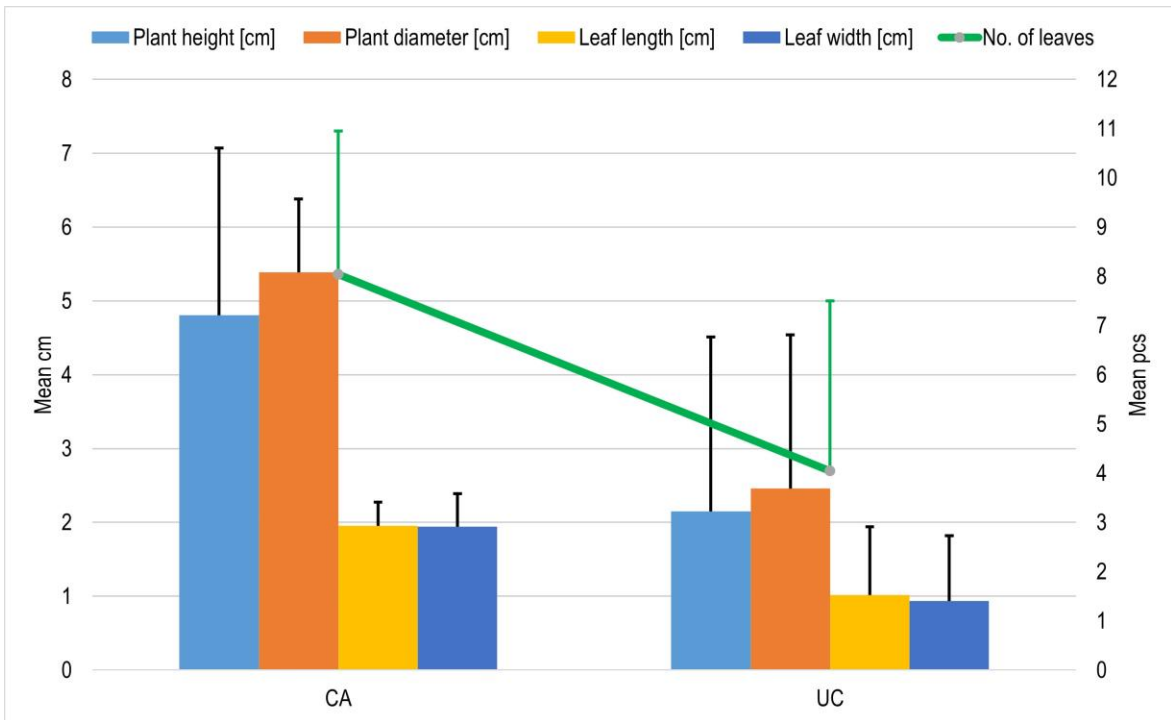


**Figure 16.:** Mean and standard deviation of Chlorophyll a + b [µg/mg] and Carotenoid [µg/mg] of *A. liliifolia* that followed three different pH levels (pH 5.6 - 5.8, pH 6.8 - 7, and pH 7.8 - 8). Different letters are for significantly different groups (Games-Howell post-hoc test,  $p < 0.05$ ).

The treatment pH 6.8 - 7 showed a statistically superior value in the case of Carotenoids content. Nevertheless, this value was statistically similar to the treatment pH 7.8 - 8 and statistically different from pH 5.6 - 5.8.

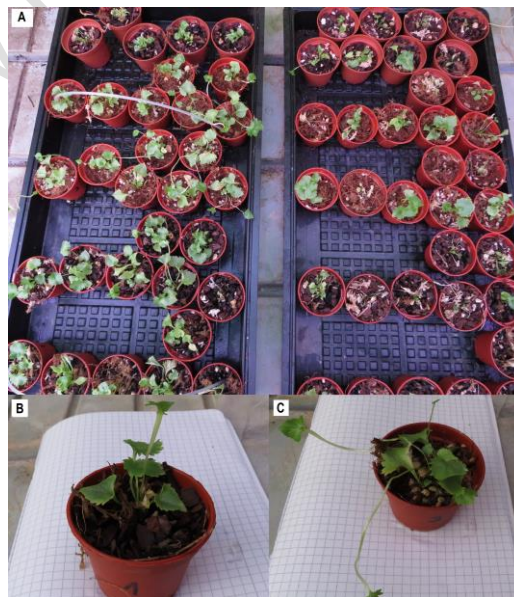
### V.3. Acclimatization

During the comparison of the type of substrate ( $S_1, S_2, S_3, S_4$ ), type of cover (CA, UC), and the interaction effect of these two factors on *Adenophora liliifolia*, two-way MANOVA did not result statistically significant for Type of substrate (Wilk's  $\lambda = 0.550$ ,  $F(15,99.782) = 1.606$ ,  $p = 0.085$ ) and the interaction of factors (Wilk's  $\lambda = 0.774$ ,  $F(15,99.782) = 0.647$ ,  $p = 0.829$ ) (Fig. 17). Post hoc tests were not performed for type of cover because fewer than three groups.



**Figure 17.:** Mean and standard deviation of Plant height [cm], Plant diameter [cm], No. of leaves, Leaf length [cm], Leaf width [cm] of *A. liliifolia* that followed two different types of cover CA (substrates covered with agronet) and UC (substrates uncovered).

Plants covered with agronet were greener and produced more leaves than uncovered ones (Fig. 18). Moreover, among covered plants, those which grow on bark had darker green leaf blades. The leaves of plants growing in coco fiber were pale, and the newly developed leaves had very long thin petioles. Newly developed leaves grown uncovered had stronger petioles, and leaves did not bend.



**Figure 18.:** Evaluation acclimatization experiment: A. CA and UC, B. S<sub>1</sub> (small pieces of pine bark, wetted sphagnum moss, perlite), C. S<sub>3</sub> (coconut fiber, sphagnum moss, perlite) (photos by Tillyné Mándy Andrea).

## VI. CONCLUSION

The different medium components and acclimatization experiments helped determine the best conditions for growing *Adenophora liliifolia ex situ*.

The first experiment indicated that the higher doses of silver nitrate caused fewer roots than the control treatment. Nevertheless, silver nitrate inhibited the unfavorable vitrification process and had a beneficial effect on the condition of the plants. Silver nitrate has been proved to be an ethylene action inhibitor and is widely used in plant tissue culture (Kumar *et al.*, 2009). In this case, a dose of up to 5 mg L<sup>-1</sup> of silver nitrate is recommended to prevent vitrification without affecting the number of roots and shoot length of the plantlets.

Additionally, the experiment showed a decreased shoot length with a dose of 20 mg L<sup>-1</sup> and a reduced number of roots with doses 10 to 20 mg L<sup>-1</sup>. Sudhersan *et al.* (2003) found media containing 40 mg L<sup>-1</sup> of silver nitrate slowed down the growth and elongation of the plantlets in four native plants from Kuwait (*Rhanterium epapposum* Oliv., *Ochradenus baccatus* Delile., *Nitraria retusa* (Forssk.) Asch., and *Lysium shawii* Roem. & Schult.). The plantlets were miniature compared to the normal plantlets and stayed longer without a single subculture. Thus, higher concentrations of silver nitrate to the culture media could be used for *in vitro* conservation, reducing the cost of plantlet production.

The second experiment with 1-Naphthaleneacetic acid (NAA) demonstrated that the growing medium ½ MS without the addition of NAA was adequate for optimal growing conditions. The control treatment caused a positive increase in all the variables linked to the experiment, like Plant height [mm], Leaf length [mm], Leaf width [mm], Root length [mm], No. of roots, and No. of shoots. In contrast, higher doses of 0.5 - 1 mg L<sup>-1</sup> indicated growth inhibition.

1-naphthaleneacetic acid (NAA) is a biologically active synthetic auxin (Olatunji & Kelley, 2021). Sudhersan *et al.* (2003) applied 0 - 0.1 mg L<sup>-1</sup> as media components for growth and rooting in different stages of native plant tissue culture to supplement the MS basal medium. Paunescu (2010) identified media MS supplemented with 0.1 mg L<sup>-1</sup> NAA and 1 mg L<sup>-1</sup> BAP as one of the best for *Campanula polymorpha*. Further studies should analyze the interaction of NAA and different concentrations of BAP.

The third experiment with coconut water revealed that from 25 to 100 ml L<sup>-1</sup>, there is a proliferation of a high number of roots, but up to 200 ml L<sup>-1</sup>, there is an inhibition of roots growing. Opposite to this, from 25 to 100 ml L<sup>-1</sup>, there is an inhibition of number of shoots, but up to 200 ml L<sup>-1</sup>, there is a proliferation of shoots growing. Coconut water contains auxin as indole-3-acetic acid (IAA), various cytokinins, GAs, ABA, Salicylic acid, vitamins, and undefined chemical components that may synergize with the other phytohormones. Cytokinins are the essential components in coconut water (Yong *et al.*, 2009).

The auxin-cytokinin hypothesis predicted that cytokinins, together with auxins, play an essential role in plant morphogenesis by controlling the formation of roots and shoots and moderating their relative growth. Also, cytokinins have a negative regulatory function in root growth whereby it suppresses cell division in plant roots (Werner *et al.*, 2001). After the experiment, a dose of 50 ml L<sup>-1</sup> is recommended for increasing the number of roots and 200 ml L<sup>-1</sup> for increasing the number of shoots on *in vitro* propagation of *Adenophora liliifolia*.

Additionally, the Chlorophyll a + b [ $\mu\text{g}/\text{mg}$ ] and Carotenoid [ $\mu\text{g}/\text{mg}$ ] content experiment confirmed a high concentration of these pigments in the control treatment of coconut water, the one that was performed the lowest response of the variables. Chlorophyll a, b, and carotenoid contents and their allocation could be an adequate indicator in evaluating plant production and environmental stress. Higher Chlorophyll a + b content, larger than 3, represents causing stress under high-light or nitrogen-lacking conditions (Sonobe *et al.*, 2020). Though, when the doses of coconut water increase, Chlorophyll a + b, and Carotenoid values decrease considerably. Cytokinins in the coconut water could have played an essential role in reducing the Chlorophyll a + b and Carotenoid values. Liu *et al.* (2020) mention that cytokinins promote plant growth and development under normal plant growth conditions and play an essential role in plant resistance to stress.

The fourth experiment in pH optimization showed a range of pH from 6.8 - 7 to 7.8 - 8 resulted in a higher plant height and number of roots than a pH of 5.6 - 5.8. Medium pH is generally adjusted to 5.8 to 6.0 for plant tissue culture; however, the medium is adjusted to a specific pH depending on the plant species used and the purpose (Shi & Yang, 2017).

Complementary data of soil samples from the natural habitat of *Adenophora liliifolia* like Dabas indicates a pH of 6.6 and CE of 1.26 mS/cm, and soil samples of Ócsa showed a pH of 6.7 and CE of 0.72 mS/cm. Thus, optimizing the medium to a pH 6.8 - 7 seems to be the best option. However, the Carotenoid content experiment evidenced a significant accumulation of this pigment in pH 6.8 - 7 treatment and no difference in Chlorophyll a + b with the other treatments. Carotenoids are auxiliary pigments in photosynthesis; they transfer the absorbed energy to Chlorophyll with an efficiency of 15% – 90%. Also, they protect Chlorophyll from the excessive light intensity. Changes in the carotenoid content within one plant mainly depend on the stage of vegetation, weather conditions, and the method of conservation (Zielewicz *et al.*, 2020).

Finally, the acclimatization experiment did not show statistically significant differences between the four substrates in all the variables. However, it was evident a better plant growth in the ones covered with agronet. Acclimatization is the last phase in which plants need to be gradually adapted to the *ex vitro* environment. This stage is determinant for the success of the *in vitro* propagation of micropropagated plantlets. In the laboratory, they are under very different conditions from when transplanted to the natural

environment (Arthur *et al.*, 2019). Further experiments with more sample sizes should be done. Unfortunately, the most suitable substrate for acclimatization of *Adenophora liliifolia* was not successfully defined.

To sum up, these experiments offer helpful information as a starting point for *in vitro* propagation of the endangered plant *Adenophora liliifolia* and its mass propagation. These plantlets are an *ex situ* stock for maintaining the *Adenophora liliifolia* population and its future repopulation of natural habitat or conservation in Botanical Gardens for long-term protection.

Liz Kelly Portocarrero Tantavilca



## VII.SUMMARY

The current study demonstrates that *Adenophora liliifolia* can be multiplied and rooted on synthetic medium, Murashige and Skoog (MS) supplemented with different doses of other plant growth regulators (PGR) and natural extracts, with good efficiency. The experiments establish a rapid and simple micropropagation system *in vitro* plantlet for future reintroduction purposes.

Moreover, PGR composition and natural extracts affect *A. liliifolia* plants growth and development. Following the results, the suitable growing medium for *A. liliifolia* was ½ MS with the following indications:

- 1) A dose of 5 ml L<sup>-1</sup> of silver nitrate is recommended for adding to *A. liliifolia* medium. The experiment resulted in many roots and high shoot length, similar to the control treatment but reduced the rate of vitrification. A high concentration of silver nitrate had a negative effect on the number of roots. Therefore, the use of a higher dosage is not recommended.
- 2) Even though the control treatment of 1-Naphthaleneacetic acid performed the best results for all the variables, 0.1 mg L<sup>-1</sup> treatment was not too lagging compared to the control group in the case of plant height.
- 3) A dose of 50 ml L<sup>-1</sup> coconut water is recommended for increasing the number of roots and 200 ml L<sup>-1</sup> for increasing the number of shoots on *in vitro* propagation of *A. liliifolia*. The higher doses of coconut water had a negative effect on the number of roots but a positive effect on the number of shoots. Moreover, the Chlorophyll a + b [µg/mg] and Carotenoid [µg/mg] content experiment confirmed a high concentration of these pigments in the control treatment of coconut water, the one that was performed the lowest results of the variables as a response to stress.
- 4) The pH optimization to a range of pH from 6.8 - 7 to 7.8 - 8 performed a significantly higher plant height and number of roots than a pH of 5.6 - 5.8. However, the Carotenoid content experiment evidenced a significant accumulation of this pigment in pH 6.8 - 7. The soil samples from the habitat of *A. liliifolia* indicated a pH of 6.65. Further investigations are required to find the optimal pH for it, with minimal plant stress.
- 5) Further acclimatization experiments implementation is required with bigger sample sizes for the correct evaluation of the optimal substrates. The experiment did not show differences between the four types of substrates in all the variables. However, it was evident a better plant growth in the ones covered with agronet.

Finally, the attractive flowers of *Adenophora liliifolia* that might have a high ornamental potential for their beauty achieved significant improvement for the *in vitro* propagation. In our study, the *in vitro* propagation and protocol improvement resulted in successful propagation and acclimatization of the species. These results are giving the starting point for further restoration programs and conservation management actions.

## VIII. ACKNOWLEDGMENTS

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Liz Kelly Portocarrero Tancovics

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## X. APPENDIX

Appx 1. Mean and standard deviation of Shoot length [mm], No. of shoots and No. of roots of *Adenophora liliifolia* that followed four different concentrations of Silver Nitrate ( $\text{AgNO}_3$ ). Means within a column followed by the same letter are not significantly different from each other at 5% level by Games-Howell test.

AgNO <sub>3</sub> concentration (mg L <sup>-1</sup> )	Variable ± sd		
	Shoot length [mm]	No. of shoots	No. of roots
Control	37.806 ± 6.645 b	4.194 ± 1.390 a	4.250 ± 3.426 c
5	36.861 ± 8.040 b	3.778 ± 1.098 a	3.972 ± 4.748 bc
10	35.111 ± 7.988 b	3.556 ± 1.229 a	1.222 ± 1.884 a
20	28.194 ± 3.808 a	3.722 ± 1.386 a	1.917 ± 2.545 ab
<b>Sign.</b>	*	ns	*

Appx 2. Mean and standard deviation of Plant height [mm], Leaf length [mm], Leaf width [mm], Root length [mm], No. of roots and No. of shoots of *Adenophora liliifolia* that followed four different concentrations of NAA. Means within a row followed by the same letter are not significantly different from each other at 5% level by Games-Howell test.

Variable ± sd	NAA concentration (mg L <sup>-1</sup> )				Sign.
	Control	0.1	0.5	1	
Plant height [mm]	49.692 ± 16.090 b	42.725 ± 13.858 b	31.591 ± 14.894 a	33.133 ± 15.875 a	*
Leaf length [mm]	16.205 ± 4.725 c	11.550 ± 4.356 b	8.682 ± 4.670 a	9.867 ± 5.350 ab	*
Leaf width [mm]	14.667 ± 4.415 c	9.975 ± 4.769 b	6.886 ± 4.244 a	6.467 ± 4.703 a	*
Root length [mm]	20.077 ± 5.918 c	14.700 ± 9.533 b	9.750 ± 6.549 a	10.911 ± 6.108 ab	*
No. of roots	10.359 ± 4.451 b	6.275 ± 4.894 a	4.409 ± 3.230 a	3.867 ± 2.801 a	*
No. of shoots	6.308 ± 1.794 c	4.575 ± 2.241 b	3.477 ± 1.836 a	2.644 ± 1.598 a	*

Appx 3. Mean and standard deviation of Plant height [mm], Root length [mm], No. of roots and No. of shoots of *Adenophora liliifolia* that followed five different concentrations of Coconut water. Means within a column followed by the same letter are not significantly different from each other at 5% level by Games-Howell's post hoc test.

Coconut water concentration (ml L <sup>-1</sup> )	Variable ± sd			
	Plant height [mm]	Root length [mm]	No. of roots	No. of shoots
Control	27.762 ± 4.516 a	18.857 ± 6.405 a	10.238 ± 3.872 a	4.095 ± 1.136 ab
25	28.048 ± 6.383 a	18.667 ± 6.583 a	11.810 ± 6.137 ab	3.524 ± 1.327 a
50	30.619 ± 4.588 a	20.286 ± 8.156 a	14.619 ± 5.463 b	3.286 ± 1.309 a
100	32.476 ± 7.012 a	21.048 ± 9.173 a	10.952 ± 2.854 ab	3.762 ± 1.091 a
200	29.952 ± 6.614 a	15.714 ± 7.818 a	8.095 ± 3.780 a	5.381 ± 2.397 b
<b>Sign.</b>	ns	ns	*	*

Appx 4. Mean and standard deviation of Plant height [mm], Leaf length [mm], Leaf width [mm], No. of roots, No. of shoots, and No. of leaves of *Adenophora liliifolia* that followed three different pH level. Means within a row followed by the same letter are not significantly different from each other at 5% level by Games-Howell's post hoc test.

Variable ± sd	pH Treatment			Sign.
	pH 5.6-5.8	pH 6.8-7.0	pH 7.8-8.0	
<b>Plant height [mm]</b>	32.417 ± 4.969 a	37.667 ± 7.446 b	38.542 ± 6.941 b	*
<b>Leaf length [mm]</b>	14.292 ± 3.057 a	15.333 ± 3.875 a	14.292 ± 2.662 a	ns
<b>Leaf width [mm]</b>	14.583 ± 3.821 a	15.125 ± 4.619 a	15.083 ± 3.717 a	ns
<b>No. of roots</b>	7.250 ± 2.982 a	10.500 ± 3.612 b	8.917 ± 3.525 ab	*
<b>No. of shoots</b>	3.667 ± 1.971 a	3.458 ± 1.215 a	3.292 ± 1.756 a	ns
<b>No. of leaves</b>	14.292 ± 5.752 a	15.500 ± 5.831 a	14.917 ± 7.879 a	ns

Appx 5. Mean and standard deviation of Chlorophyll a+b [ $\mu\text{g}/\text{mg}$ ] and Carotenoid [ $\mu\text{g}/\text{mg}$ ] of *Adenophora liliifolia* that followed four different concentrations of Coconut water. Means within a column followed by the same letter are not significantly different from each other at 5% level by Tukey HSD test.

Coconut water Treatment (ml L <sup>-1</sup> )	Variable $\pm$ sd	
	Chlorophyll a+b [ $\mu\text{g}/\text{mg}$ ]	Carotenoid [ $\mu\text{g}/\text{mg}$ ]
Control	1.224 $\pm$ 0.229 b	0.234 $\pm$ 0.038 b
50	0.522 $\pm$ 0.251 a	0.120 $\pm$ 0.040 a
100	0.312 $\pm$ 0.050 a	0.085 $\pm$ 0.012 a
200	0.215 $\pm$ 0.038 a	0.070 $\pm$ 0.010 a
<b>Sign.</b>	*	*

Appx 6. Mean and standard deviation of Chlorophyll a+b [ $\mu\text{g}/\text{mg}$ ] and Carotenoid [ $\mu\text{g}/\text{mg}$ ] of *Adenophora liliifolia* that followed three different pH level. Means within a column followed by the same letter are not significantly different from each other at 5% level by Games-Howell post-hoc test.

pH Treatment	Variable $\pm$ sd	
	Chlorophyll a+b [ $\mu\text{g}/\text{mg}$ ]	Carotenoid [ $\mu\text{g}/\text{mg}$ ]
pH 5.6-5.8	0.650 $\pm$ 0.167 a	0.134 $\pm$ 0.026 a
pH 6.8-7.0	0.850 $\pm$ 0.109 a	0.184 $\pm$ 0.009 b
pH 7.8-8.0	0.895 $\pm$ 0.206 a	0.173 $\pm$ 0.034 ab
<b>Sign.</b>	ns	*



Appx 7. Mean and standard deviation of Plant height [cm], Plant diameter [cm], No. of leaves, Leaf length [cm], Leaf width [cm] of *Adenophora liliifolia* that followed four different substrates (S<sub>1</sub>, S<sub>2</sub>, S<sub>3</sub>, S<sub>4</sub>), covered with agronet (CA) and uncovered (UC). Means within a column followed by the same letter are not significantly different from each other at 5% level by Games-Howell post-hoc test.

	<b>Variable ± sd</b>	<b>Mean</b>	<b>± sd</b>
<b>CA</b>	Plant height [cm]	4.804	± 2.264
	Plant diameter [cm]	5.388	± 0.990
	No. of leaves	8.042	± 2.911
	Leaf length [cm]	1.954	± 0.319
	Leaf width [cm]	1.942	± 0.446
<b>UC</b>	Plant height [cm]	2.150	± 2.361
	Plant diameter [cm]	2.458	± 2.081
	No. of leaves	4.046	± 3.454
	Leaf length [cm]	1.017	± 0.922
	Leaf width [cm]	0.933	± 0.885

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## DECLARATION

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*In vitro* propagation and conservation of *Adenophora liliifolia* Ledeb. ex A. DC., an endangered and potential ornamental species

MSc in Horticultural Engineering

*Adenophora liliifolia* Ledeb. ex A. DC. belongs to the *Campanulaceae* family, and it is an endangered species in Hungary with highly ornamental potential for its showy flowers. Although the plants have been protected in different National parks in several countries, there is not enough knowledge about the massive propagation of this species. Therefore, we studied the effect of plant growth regulators (PGR) and natural extracts in the *in vitro* propagation and the acclimatization process of this species.

The medium experiments were carried out in the Department of Floriculture and Dendrology laboratory and the acclimatization experiment was held in the ELTE Botanical Garden.

The medium experiments studied the effect of silver nitrate in different doses (Control, 5 mg L<sup>-1</sup>, 10 mg L<sup>-1</sup>, 20 mg L<sup>-1</sup>), 1-Naphthaleneacetic acid (Control, 0.1 mg L<sup>-1</sup>, 0.5 mg L<sup>-1</sup>, 1 mg L<sup>-1</sup>), coconut water (Control, 25 ml L<sup>-1</sup>, 50 ml L<sup>-1</sup>, 100ml L<sup>-1</sup>, 200 ml L<sup>-1</sup>) and pH gradient (pH 5.6 - 5.8, pH 6.8 - 7, pH 7.8 - 8). Additionally, experiments were established to determine Chlorophyll and Carotenoids content in the coconut water and pH gradient treatments. One-way MANOVAs were performed for each experiment.

Four types of substrates: S<sub>1</sub> (small pieces of pine bark, wetted sphagnum moss, perlite), S<sub>2</sub> (small pieces of pine bark, sphagnum moss, small pieces of zeolite stone), S<sub>3</sub> (coconut fiber, sphagnum moss, perlite), S<sub>4</sub> (coconut fiber, sphagnum moss, zeolite) and two types of cover: CA (covered with agronet), UC (uncovered) were used for the establishment of the acclimatization experiment. Two-way MANOVA was performed in the experiment.

Moreover, PGR composition and natural extracts affect *A. liliifolia* plants growth and development. Following the results, the suitable growing medium for *A. liliifolia* was ½ MS with the following indications:

- 1) A dose of 5 ml L<sup>-1</sup> of silver nitrate is recommended for adding to *A. liliifolia* medium. The experiment resulted in many roots and high shoot length, similar to the control treatment but reduced the rate of vitrification. A high concentration of silver nitrate had a negative effect on the number of roots. Therefore, the use of a higher dosage is not recommended.

- 2) Even though the control treatment of 1-Naphthaleneacetic acid performed the best results for all the variables, 0.1 mg L<sup>-1</sup> treatment was not too lagging compared to the control group in the case of plant height.
- 3) A dose of 50 ml L<sup>-1</sup> coconut water is recommended for increasing the number of roots and 200 ml L<sup>-1</sup> for increasing the number of shoots on *in vitro* propagation of *A. liliifolia*. The higher doses of coconut water had a negative effect on the number of roots but a positive effect on the number of shoots. Moreover, the Chlorophyll a + b [µg/mg] and Carotenoid [µg/mg] content experiment confirmed a high concentration of these pigments in the control treatment of coconut water, the one that was performed the lowest results of the variables as a response to stress.
- 4) The pH optimization to a range of pH from 6.8 - 7 to 7.8 - 8 performed a significantly higher plant height and number of roots than a pH of 5.6 - 5.8. However, the Carotenoid content experiment evidenced a significant accumulation of this pigment in pH 6.8 - 7. The soil samples from the habitat of *A. liliifolia* indicated a pH of 6.65. Further investigations are required to find the optimal pH for it, with minimal plant stress.
- 5) Further acclimatization experiments implementation is required with bigger sample sizes for the correct evaluation of the optimal substrates. The experiment did not show differences between the four types of substrates in all the variables. However, it was evident a better plant growth in the ones covered with agronet.

Finally, the attractive flowers of *Adenophora liliifolia* that might have a high ornamental potential for their beauty achieved significant improvement for the *in vitro* propagation. In our study, the *in vitro* propagation and protocol improvement resulted in successful propagation and acclimatization of the species. These results are giving the starting point for further restoration programs and conservation management actions.

The experiments establish a rapid and simple micropropagation system *in vitro* plantlet for future reintroduction purposes. These plantlets are an *ex situ* stock for maintaining the *Adenophora liliifolia* population and its future repopulation of natural habitat or conservation in Botanical Gardens for long-term protection.