CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Tropical AgriSciences

Department of Crop Sciences and Agroforestry

Czech University of Life Sciences Prague

Faculty of Tropical AgriSciences

***In vitro* propagation of *Darlingtonia californica* and assessment of genetic stability in regenerants using molecular markers**

Master thesis

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Supervisor:

Ing. Iva Viehmannová, PhD

Author:

Bc. Anežka Houšková

Declaration:

I confirm that master thesis named "*In vitro* propagation of *Darlingtonia californica* and assessment of genetic stability in regenerants using molecular markers" was originally made as a result of my own research and work at Czech University of Life Sciences Prague (CULS Prague). I also declare that all resources are properly cited and all of them are noted in list of references in given document. I honestly declare that I have not used any other sources in addition to those noted. I do agree with use of my master thesis for study purposes within CULS Prague.

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Houšková Anežka

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Abstract

The goal of this master thesis was a development of efficient protocol for direct morphogenesis of the carnivorous pitcher plant *Darlingtonia californica* Torr. from family Sarraceniaceae. For *in vitro* propagation and *in vitro* rooting, MS medium supplemented with d ifferent concentrations of plant growth regulators was used. Totally, twelve media for shoot formation were tested (1/2 MS with zeatin or BAP alone or in combination with NAA). For rooting, six media were tested (1/3 MS with NAA and 1/4 MS with different concentrations of NAA). The highest production of new shoots was achieved on 1/2 MS supplemented with 0.5 $mg \cdot l^{-1}$ zeatin and 0.1 $mg \cdot l^{-1}$ NAA. The rooting media did not provide satisfactory result and thus, further research is needed. The regenerated plants were tested for ploidy stability by flow cytometry which revealed no change. As a molecular marker for assessment of genetic stability, inter single sequence repeat was used. Totally, ten samples and one control were tested by twelve primers generating 605 scorable bands with all the bands being monomorphic. It can be concluded, that the protocol could be used for efficient *in vitro* propagation of *Darlingtonia* but a protocol for rooting is yet to be carried out.

Keywords: carnivorous plant, *Darlingtonia*, flow cytometry, *in vitro*, ISSR, micropropagation, plant growth regulators

Abstrakt

Cílem této diplomové práce bylo vytvoření efektivního protokolu pro přímou morfogenezi masožravé rostliny darlingtonie kalifornské (*Darlingtonia californica* Torr.) z čeledi *Sarraceniaceae*. Pro mikropropagaci a zakořeňování bylo použito MS médium obohacené o různé koncentrace růstových regulátorů. Celkem bylo testováno dvanáct médii pro tvorbu odnoží (1/2 MS buď pouze s přídavkem zeatinu či BAP, nebo v kombinaci s NAA v koncentraci 0,1 $mg \cdot l^{-1}$). Pro zakořeňování bylo testováno šest médií (1/3 MS s NAA a 1/4 MS s různými koncentracemi NAA). Nejvíce nových odnoží bylo vytvořeno na 1/2 MS médiu obohaceném o 0,5 $mg \cdot l^{-1}$ zeatinu a 0,1 $mg \cdot l^{-1}$ NAA. Média pro zakořeňování neposkytla uspokojující výsledky a je nezbytné provést další výzkum v této oblasti. Regenerované rostliny byly testovány z hlediska ploidie pomocí průtokové cytometrie, která neodhalila žádnou změnu. Jako molekulární markery pro stanovení genetické stability byly použity “inter simple sequence repeat” (ISSR) primery. Celkem bylo testováno deset získaných regenrantů včetně kontrolního vzorku za použití 12 ISSR primerů, které vytvořily celkově 605 dobře reprodukovatelných bandů a všechny byly monomorfní. Lze shrnout, že prezentovaný protokol lze použít pro efektivní *in vitro* množení druhu *Darlingtonia californica* avšak je třeba nalézt vhodný protokol pro zakořeňování.

Klíčová slova*:* *Darlingtonia*, *in vitro*, ISSR, masožravé rostliny, mikropropagace, průtoková cytometrie, regulátory rostlinného růstu

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List of abbreviations:

2, 4-D	2, 4-dichlorophenoxyaceticacid
BAP	6-benzylaminopurine
Chloroform-IAA	Chloroform-isoamylalcohol
BSA	Bovine serum albumin
CTAB	Cetyl trimethyl ammonium bromide
CULS Prague	Czech University of Life Sciences Prague
DNA	Deoxyribonucleic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
ISSR	Inter single sequence repeat
LS	Linsmainer and Skoog (1965) medium
MAS	Marked assisted selection
MS	Murashige and Skoog (1962) medium
NAA	α-naphtylacetic acid
PAA	Phenyl acetic acid
PCR	Polymerase chain reaction
PGR	Plant growth regulator
RAPD	Random amplified polymorphic DNA
TE buffer	Tris (tris(hydroxymethyl)aminomethane) and EDTA (ethylendiaminetetraacetic acid) buffer

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1. Introduction

The Californian Cobra Lily (*Darlingtonia californica* Torr.), originating from very specific area of California in United States, is pitcher carnivorous plant from family Sarraceniaceae with very characteristic anatomy, which confers it its name. It is well known among collectors for whose it is particularly difficult plant to cultivate but popular despite its reputation.

Unfortunately the Cobra Lily is not easy to propagate by mean of natural way since its seeds are rapidly losing its germination ability and natural vegetative propagation via stolons is slow. Use of biotechnologies provides a wide range of tools how to deal with such problems. I*n vitro* direct morphogenesis could be optimal for rapid multiplication of true-to-type regenerants since the risk of genetic variability is minimized in this method.

Recently, *Darlingtonia californica* has been object of ecology studies, but no *in vitro* technologies have been optimized yet.

Therefore, the goal of this work was to develop an efficient protocol of *in vitro* propagation, which could be used for production of high amount of good quality, uniform plants. To assess the clonal fidelity of adventitious shoots, molecular markers inter simple sequence repeat and flow cytometry was used.

2. Literature review

2.1 Pitcher carnivorous plants

Darlingtonia californica is endemic carnivorous plant with patchy distribution in coastal Oregon and northern California, USA (Fashing, 2004). By its ecology, *Darlingtonia* belongs to the group of pitcher carnivorous plants (Pietropaolo and Pietropaolo, 1996).

Darlingtonia belongs to family Sarraceniaceae together with genera *Heliamphora* and *Sarracenia.* Common feature of the family are leaves, which are modified to form hollow vessels. They are ordinarily referred as pitchers and serves for capturing the prey. From this feature the whole family is called “pitcher plants”.

Carnivorous plants of family Sarraceniaceae were probably well known to native Americans before discovering the continent by Christopher Colombo. One of the first known illustrations was published in 1576 in De L'Obels's, *Nova Stirpium Adversia*. Plants of *Sarracenia purpurea* had been sent to a Mr. Tournefort in Europe by Canadian physician, Dr. M. S. Sarrazin. To honour his name, the whole genus was named after him. However, the carnivory was confirmed by Higley (1885) who demonstrated absorption through the pitcher walls by *Sarracenia* and in 1885 the digestive enzymes were discovered by Zipperer in the plants' secretions and practical demonstration of digesting capability was demonstrated by Hepburn in 1918.

2.2 History and discovery

All above-mentioned pitcher plants have homeland in North America. They can be found in bogs, swamps, low wetlands, open pine lands and in lower extend in wooded areas too. They inhabit soil poor on nutrients and organic matter and vegetation of these areas are with strikingly different physiognomies (Alexander *et al.*, 2006). These serpentine soils occupy 1.5% of California but harbours 12.5% of state's endemic plants (Safford *et al.*, 2005). However, *Darlingtonia* was discovered bit later than other plants from family Sarraceniaceae, in 1841, by Scottish nurseryman and botanist William Dunlop Brackenridge. During the expedition of captain Wilkens to California he found the new plant species near Mount Shasta. According to old story, he discovered the plant while being pursued by Native Americans (Rice, 2005).

The botanical description was done in 1853 by American botanist John Torrey (Torrey, 1853) who concluded that the differences from other Sarraceniaceae species are sufficient distinguishable to claim *Darlingtonia* as a new genus. He named the plant for the honour of his friend, outstanding biologist William Darlington from Pennsylvania (Pietropaolo and Pietropaolo, 1996). Unfortunately, according to international binomial nomenclature the name was not valid so it had to been renamed to *Chysamphora* in 1891 by professor Greene from University in Berkeley. But the old name was already widely used therefore the International botanical congress in 1954 decided for using of the older original name. The plant could proudly deserve also name after American amateur botanist Rebecca Merritt Smith Austin who was fascinated by the plant and dedicated it part of her life research (Žáček, 2008; Austin, 1880).

The common names are Cobra Lily and California pitcher plant and in some literature could be also called deer licks (Rice, 2005).

2.3 Origin and geographical distribution

The origin of the Cobra Lily is unique for quite restricted area in two west central states in United States, nominally south west Oregon and as its name prompts also Northern California (Pásek, 2013).

The distribution is virtually copying the distribution of serpentine rocks in these areas but they are not completely identical. According to Alexander *et al.* (2006) the serpentine rocks are compositions of mineral serpentine. That is a group of minerals, with the chemical formula $Mg_3Si_2O_5(OH)_4$ forming serpentine rocks when aggregating. However more accurate name is “ultramafic rock” for geologists. These minerals are widely distributed in western North America.

The range of altitudes where it grows is wide ranging from sea level up to high mountains of around 2800 meters above sea level and the plants are cover by the snow in winter. On the other hand the lowland vegetation is hardly ever exposed to even weak frost. Therefore *Darlingtonia* has high aptitude to adapt in different ecological conditions (Juniper *et al.*, 1989).

The area of distribution of California pitcher plant is territory with humid rainy winters and dry periods during summers. The dry period could last from one to four months and generally more north and in higher altitudes the shorter the dry period is (Trappe and Gerdemann, 1974). Annual precipitations are very different in the area Cobra Lily's distribution ranging from annual precipitation of 500 to 2000 mm in average. However *Darlingtonia* is not tolerant to low humidity because even during the dry period the geographical position close to the ocean is bringing the moisture (DeBuhr, 1993).

Similar situation is with temperature when the vegetation of high mountains experience deep freeze (up to –33 ºC) and vegetation of lowlands has average temperature in coldest month 1 ºC. However the plant is not tolerant to high temperatures in a long term. The Cobra lily is not subjected only to macroclimate but the microclimate is of the same or even higher importance. They often grows around streams and running water elsewhere in marches and swamps with peaty soils or gravel alluvial plains created by weathering of serpentines and banks of lakes (Alexander *et al.*, 2006). The physiological mechanism and evolutionary benefits of this discrepancy are not fully understood and need to be studied more (Adlassnig *et al.*, 2005).

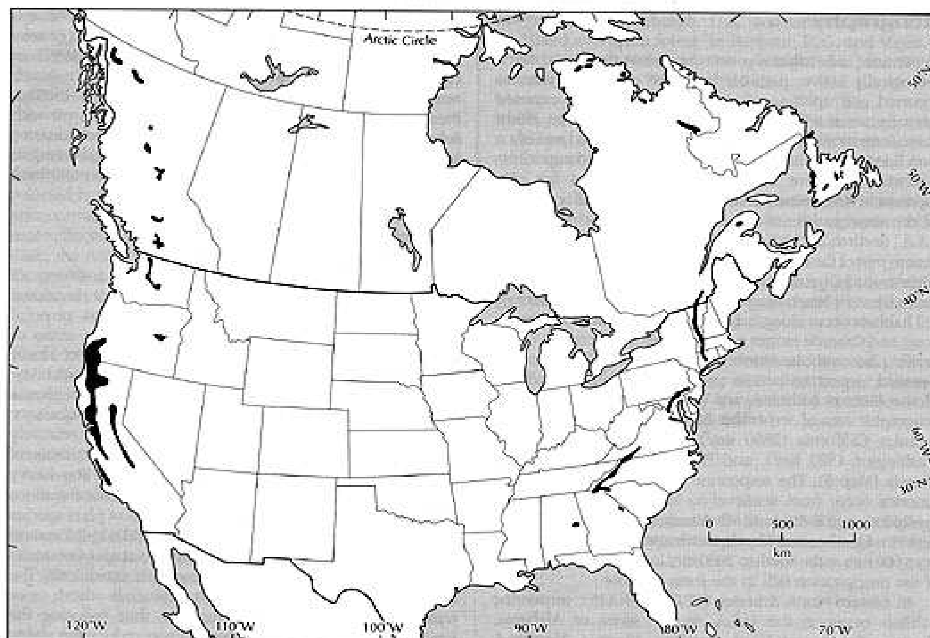

Figure 1 Distribution of ultramafic rock areas corresponding with distribution of *Darlingtonia californica* on west coast (source: Davis *et al.*, 1997)

2.4 Taxonomy

Darlingtonia is the only species of its genus belonging to the same family as genus *Sarracenia* and *Heliamphora.* Even that the pitcher plants from genus *Sarracenia* look very similar the number and morphology of chromosomes is different. *Darlingtonia* has 2n=30, *Heliaphora* 2n=42 and *Sarracenia* 2n=26. The chromosomes of *Darlingtonia* are almost two times smaller than other pitcher plants, which are the closest relatives. This support Torrey's decision of putting *Darlingtonia* in new genus (Studnička, 1982).

Most recently, Ellison and Farnsworth (2012) studied phylogeny and biogeography of family Sarraceniaceae (Figure 2). They based their study of phylogeny on seven mitochondrial, nuclear and plastid loci in spite to discovered unusual disjunctive geographical distribution. All their analysis supported above-mentioned monophyly of *Darlingtonia, Sarracenia* and *Heliapmhora*. Furthermore they deducted that during the Eocene Sarraceniaceae became widespread in the America by migrating from South to North America via a discontinuous landmass in the Antilles region that appears to have begun in the middle Eocene. Seeds of *Darlingtonia* are hydrophobic and can disperse longer distances by skimming across water surfaces (Ellison and Parker, 2002). This characteristics together with rapid population growth rate could have led to its spread beyond 10,000 km within 15 million years.

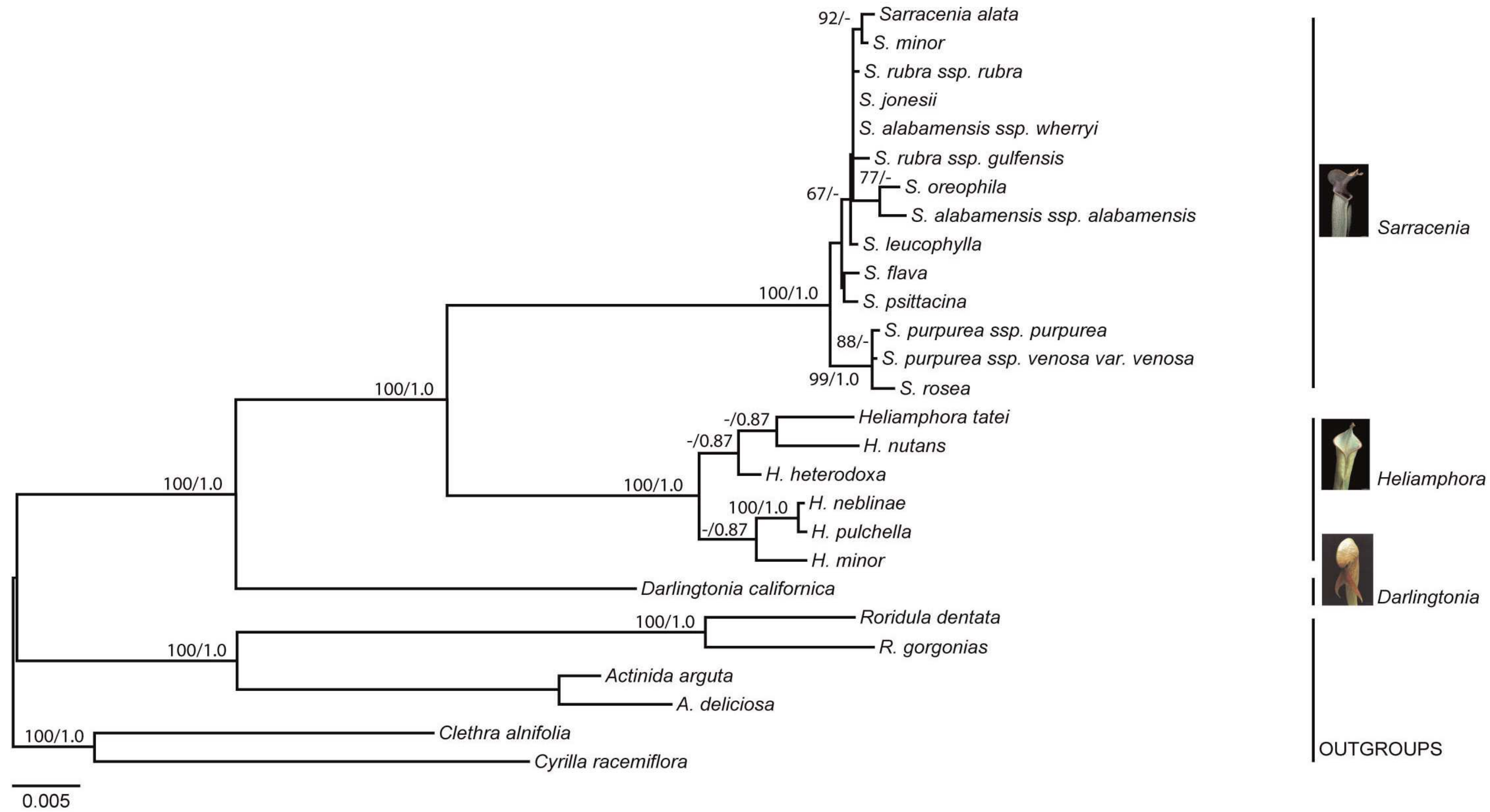

Figure 2 Maximum likelihood phylogeny of Sarraceniaceae based on plastid, nuclear, and mitochondrial data combined. (*Sarracenia purpurea* var.*montana* was excluded, with pt-plasmid, nu-nuclear mt-mitochondrial DNA) (source: Ellison and Parker*,* 2012).

2.5 Botanical description and morphology

Darlingtonia plants are herbaceous perennials with leaves growing upright and forming hollow pitchers that terminate in a dome. The domed pitchers arise from a fibrous rooted rhizome. Matured plants send out stolons from rhizomes to develop new plants (Figure 3). The pitchers twist during the development so that the opening of the pitcher is facing outward from the centre of the plant. A typical plant has from 5 to 15 leaves and usually also one scape bearing a solitary flower (Pietropaolo and Pietropaolo, 1996).

The pitchers can reach from 50 to 100 cm in natural habitat and terminate in a dome whose opening is to the front and below the dome. The leaves are growing from shallowly laid rhizome. On its end the leaves are forming a rosette of 5 to 15 leaves (Švarc, 2003). Leaves are modified (episcidiate) to form so called pitcher, (Figure 4, annex Figure 13) (Franck, 1974; 1976). They are normally produced every 2-4 weeks throughout the growing season which in general is from April/May to September/October. The leaves senesce over the winter meaning their lifespan is approximately 6 months or less (Ellison and Farnsworth, 2005). The pitches are different than other pitcher plants. They have a prominent almost spherical “hood” with a “mouth” at the base of the hood that faces downward. From the far edge of the mouth hangs a “fish tail appendage” (Ellison and Farnsworth, 2005). The hood of 12 to 15 cm in diameter terminates the pitchers and the mouth at the base has around 3 cm in diameter (Studnička, 1992). The leaf is lined from the base to the top with a lamellar wing, a typical attribute of Sarraceniaceae. Allometry of the tube, hood and fishtail appendage differs between seedlings (non-feeding) and adult (feeding) pitchers and are more or less consistent within life stages (Franck, 1976).

According to Pietropaolo and Pietropaolo (1996) the juvenile leaves produced by seedling, by side shoots from a rhizome and by cutting lack the forked appendage and dome (called also fenestration or areols). The areols make illusion of lightened space for trapped insect but it is impossible for it to get back due to the hairs. The hairs are pointed down and get denser in the basal part of pitches (Švarc, 2003).

The flowers of *Darlingtonia* are very similar to the flowers of its closest relatives, Sarraceniaceae (Studnička, 1984). They appear in scapes reaching to 100 cm. The general flower formula of *Darlingtonia* is following: ∗♀♂ K5 C5 A∞ $\underline{G(5)}$ (Srba, 2013). The flower buds are formed in the rosette centre during the cold winter months. In the spring the

buds develops to tall scapes. In May, a red solitary flower blooms in a pendulous position at the tip of o the tall scape (Elder, 1994).

The flower (Figure 5) has five purple-pink petals, which hang from the base of the dangling flower. The tip of each petal comes together to form a slightly elongated sphere. Each petal has a small notch on each side two thirds of the way down from the attached base. When the corolla sphere is formed, each adjacent notch pair creates one circular opening, five in all. The petal has pale yellow colour and is heavily line with red veins which makes the flower appear bright red (Elder, 1994; Studnička 1984). Five elongated yellow sepals softly overhang the red corolla. Ovaries which are bell-shaped hang at the base inside the corolla (Figure 4, annex Figures 11, 12).

Figure 3 *Darlingtonia californica* plant with young plant developing from stolen (source: Pietropaolo and Pietropaolo, 1996)

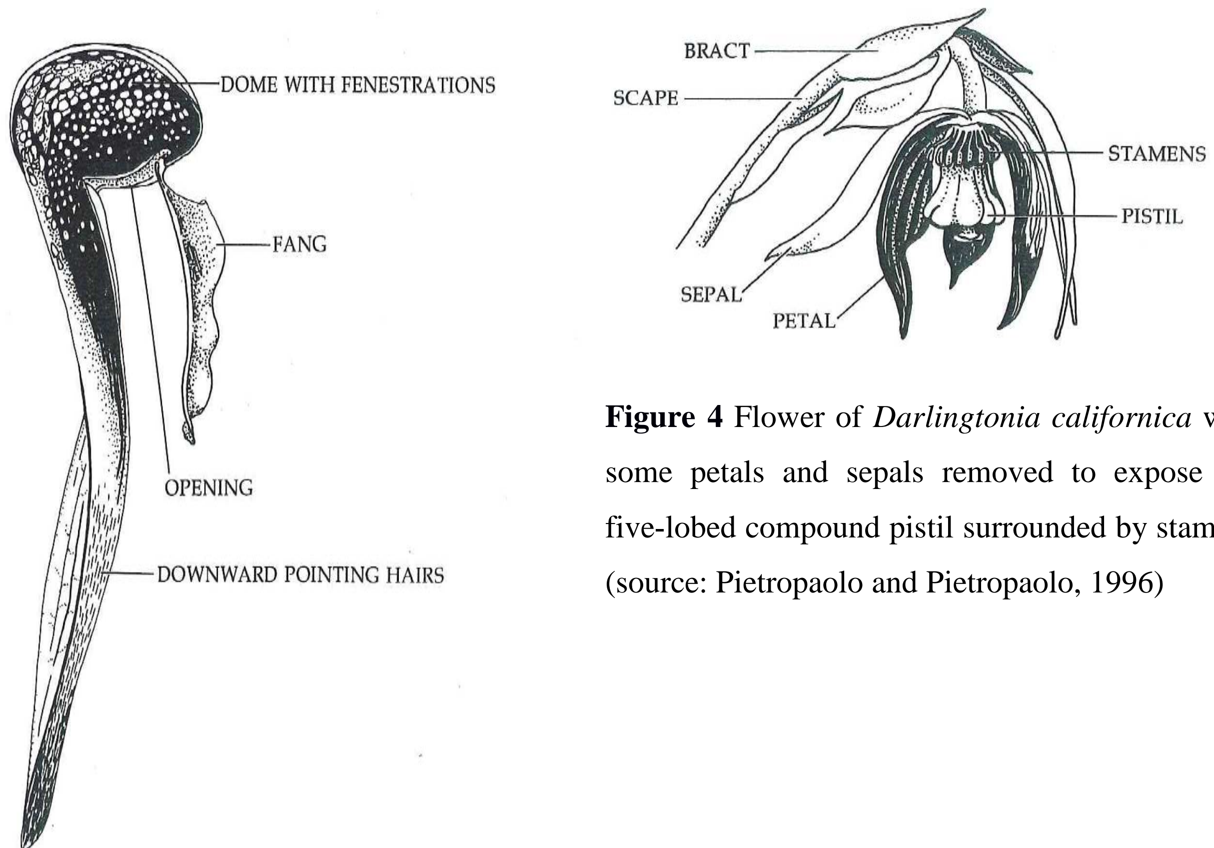

Figure 4 Flower of *Darlingtonia californica* with some petals and sepals removed to expose the five-lobed compound pistil surrounded by stamens (source: Pietropaolo and Pietropaolo, 1996)

Figure 5 Longitudinal section of *Darlingtonia californica* pitcher. The downward pointing hairs hinder prey from climbing out of the pitcher. Insects trying to fly through one of the transparent areas find themselves hitting the wall of the leaves and fall to the bottom of the pitcher (source: Pietropaolo and Pietropaolo, 1996).

The fruit is ovoid from 2.5 to 4.5 cm. The flower scapes become papery and abscise as the ovary of the flower matures into a dry fruit containing seeds (annex Figure 13). The seeds are numerous, brown to reddish brown and appear in capsules of size around 2 mm (Pietropaolo and Pietropaolo, 1996). Matured seed are cover with visible hairy trichomes. They easily flow on water and are hydrophobic and even after few days in water they do not merge (Švarc, 2003). That helps the seeds to disperse by numerous streams, which are in Cobra Lily's habitat. The seed set naturally occurs in September and the seeds germinate next spring. The juvenile leaves are tubular with a narrow pointed tip (Elder, 1994).

There is a contrast of pace of growth and size of Cobra Lily because for its size, *Darlingtonia* is rather slow grower because it takes two to three years for juvenile, pointed-ended leaves to develop the characteristic of mature pitchers. And additional few years are required for the plant to flower. As the plant grows it develops larger leaves every year until it reaches maturity in 7-10 years from the little seedling (Romanowski, 2002). That is the main reason why *Darlingtonia* reproduce more likely vegetatively then generatively. The mature plants often produce a new growth point in the rosette centre and consequently grow in two plants. Furthermore Cobra Lilly habitually produces quite long stolons or underground runners from the thick rhizomes and these stolons develop into a new plant (Elder, 1994). The asexual propagation often results in dense growth in very well distinguishable characteristic population as typically seen in the wild population in their habitat (Trappe and Gerdemann, 1974).

2.6 Life strategy and ecology

Darlingtonia californica as a carnivorous plant has developed an effective mechanism how to trap various insect to enhance their mineral intake because in general, carnivorous plants grow on poor soils low in nutrition.

The prey is attracted to the “head” of pitcher based on principle of reflectors, which is an important mechanism for insect for finding the nourishment in flowers. The head with numerous aerols is lighted and can be yellowish green or reddish green. The secretion of nectar evokes illusion of real flowers. Captured insect is guided deeper in the pitcher by tenacious down-pointing hairs, which grow in the places between aerols. The insect soon realizes its mistake and tries to escape but the surface is passable only downwards. The lightened “windows” also help to confuse the captured prey while the real entrance is wisely

hidden thanks to its special anatomy. Finally insect has to take the way of the “least resistance” to the narrowing pitcher. The surface is covered with short cuspidate out-shots of cells. That is a very similar mechanism as in the closest relatives, Sarraceniaceae (Studnička, 1992).

The lower part of pitcher has long hairs and contains a liquid where the preys drown in. Contrary to plants with similar hunting mechanism, the monkey cups or so called tropical pitcher plants (*Nephentes*). The inner liquid does not have higher wetting power. The liquid appears already in juvenile pitchers and in fully developed pitchers cannot be replenished by rainfall because it is covered by “headed end”. It is clear then, that the liquid is produced by inner tissue of pitcher even if no specially differentiated cells have been found. The secretion is probably supported by captured prey (contrary to other Sarraceniaceae) (Schnell, 1976, Slack, 1979). This dependency on microorganisms is favourable because the traps can survive much longer in comparison with other carnivorous plants. They commonly live 2 years or even longer if they are not killed by the freeze in winter (Studnička, 1982).

Very interesting is also the biology of pollination. A pollinator enters the corolla through one of the circular openings. Due to the flower morphology described above, the pollinator is bound to brush the stigma immediately upon the entry and deposit the pollen collected from previously visited flower. And when the insects seek for the nectar in the interior of the corolla, it collects new pollen. For leaving the flower, the pollinator just slides down the ovary slope and exit the flower through one of the circular opening or by pushing the petal tips. This strategy encourages the cross-pollination and reduces the chance of selfing (DeBuhr, 1974). But any field observation does not support this theory. The unidentified pollinator is escaping the discovery for the past 150 years. Schnell (2002) says pollination biologist will one day identify a bee to be the yet-unknown pollinating agent as for *Sarracenia* flowers in the East. Austin (1880) was suspecting ever-ubiquitous spider to be a pollinator because they are very common in Cobra Lilies fields and indeed it is rare to find spider free flower. Rondeau (1995) points out the total lack of investigation on nocturnal insects and some entomologist believe that the strong unpleasant odour of the flower clearly suggest the pollination by flies (Rondeau, 1995).

The similarity of flower and its own traps, which was observed by field biologists, is posing a question if the dual attractiveness also extends to the insects meaning the pollinator/prey paradox may arise. Are the flowers and the pitchers supposed to attract the

same insect? Because if they are, they compete for same visitors leading to pollinator/prey dilemma. However the average distance between the height of flower and mature pitch is around 20 cm. This offers some spatial separation between trapping and pollination zones. Another partial separation is by time because a field observation shows a relatively limited number of functional traps are remaining at the time of anthesis. This offers one-month isolation so the paradox is lowered (Nyoka and Ferguson, 1999).
Other interesting ecological characteristic is the compass nature of *Darlingtonia.* This feature was observed for the first time by Austin (1880). According to her observations the first two large leaves in the spring would face the north-south direction and the next two would emerge in the east-west direction. Schnell (2002) noted that his cultivated plants obeyed this compass rule. When he turned the pot 90 degrees the plant produced the leaves according to the old orientation in the first year but adjusted to the new orientation the following year.

2.7 Propagation

In nature, two ways of propagation are common, by the seeds or by the stolon. The seeds are highly hydrophobic and do not absorb water. That helps them to be distributed in their natural habitat by streams and flowing water (Švarc, 2003). Strangely, they are covered by trichomes, which are feature of seeds distributed by animals. In nature they come to maturity in autumn and hibernate during the winter. They don't success to germinate without the cold period (Studnička, 1982).

The vegetative propagation by stolons is also very common. Fires often strike the area where D. californica grows. The treatment of very high temperatures destroys the upper part of plant but it would eventually survive underground in form of stolons. Furthermore, the fires destroys the surrounding vegetation and thus lower the competition and prepare the suitable conditions pro germinating and rooting of new seedlings (Jules *et al*., 2001).

2.8 Cultivars

There are not many cultivars of *Darlingtonia californica* described. One of the most important is full green cultivar “Othello” which was discovered by Meyers-Rice in spring 1997. This plant cultivar differs from others, which are red-pigmented. The cultivar was found in the Californian Sierra Nevada. It is an anthocyanin-free form of *Darlingtonia*

californica. It is characterized by one feature-a lack of red pigment in the leaves and flowers. This cultivar is of considerable interest to horticulturists because its numerous pitchers are large, well formed and the plant liberally produces new plants by stolons (Meyers-Rice, 1998). It is also only registered cultivar of Cobra Lily in The Carnivorous Plant Database (ICPR, 2013).

The collectors distinguish different colour variant although it is not official.

Amongst the trait which differs are:

Green fangs: an overall green plant

Red-edge fangs: similar to all green plant but has pigmentation in the fangs

Blush fangs: has a blush of red in the fangs

Crimson fangs: the fangs are uniformly pigmented to deep red and the rest of plant is deep green

Red keel: the pitcher is red, an intermediate between "crimson fangs" and "crimson pitcher"

Crimson pitcher: entire pitcher is intensely coloured deep red

A newest taxonomic revision of the Sarraceniaceae was done in 2011 by McPherson and Schnell who recognize two intra-specific taxa of *D. californica:*

D. californica f. *californica*

D. californica f. *viridiflora*

2.9 Home and greenhouse cultivation

It is said that *D. californica* is one of the most difficult carnivorous plant from North America to cultivate and it is not going to be widespread home ornamental plant. However it is very popular among carnivorous plant lovers and collectors who consider the cultivation of *Darlingtonia* as a challenge and who share experiences about care of this beauty in diverse internet forums, clubs, associations etc.

Pots: The clay pots are most suitable because they are cooled down by water evaporation of its porous surface. Plastic containers are less convenient but could be used as well. The techniques how to cool them is to wrap the container in the aluminium foil which would reflect the sun light or they can be placed into bigger containers filled with live bog moss. (Studnička, 1982; Švarc, 2009).

Substrate: As a substrate the mixture of peat, and perlite, keramzite or crushed granite and the topsoil is covered around the plants by bog moss (Švarc, 2009).

Light: For home conditions the artificial light is highly recommended. The combination of special fluorescent lamp for plants with common white fluorescent lamp and controlling by switch clock gives the best results especially in cold basements and caves or fully unnatural conditions. Otherwise the plant likes very high luminosity (Studnička, 1982)

Temperature: The temperature of the soil is even more important than the temperature of air because in nature the roots are continuously cooled down by flowing water. Ideally in hotter period should be around 18 °C. In hot summer it can be achieved by often overflowing or adding of a piece of ice on the surface, eventually by wrapping the whole pot into the peat. Well-acclimatized plants can support up to 30 °C in summer and from 0 °C to 10 °C in winter. In some regions *Darlingtonia* could be grown outside whole year or placed in cold greenhouse (Pietropaolo, 1996; Studnička, 1982; Švarc, 2009).

Moisture: In nature where the plant occurs the average humidity is 60-80% all year. Thus it is very important to keep them in similar conditions but the roots shouldn't be merged in water continuously. Generally, the very high air humidity together with unusually low substrate temperature is the most suitable (Pietropaolo, 1996; Studnička, 1982; Švarc, 2009).

Propagation: Could be done by seeds, which are sown immediately after harvest in autumn. They are just gently pushed into the surface of the substrate. The substrate should be composed of peat and rough sand 1:1 and they placed to the fridge. They should be kept there 6 to 8 weeks in temperature around 0 °C. The seedlings start to germinate after 2 months after end of cold period. Very important is not to cross the maximum temperature of 15 °C. The growth of seedlings is very slow and first prey pitchers occur the third year after sowing (Studnička, 1982).

Easier way of propagation is vegetative way by division of naturally produced stolons which is done in spring during the transplanting (Švarc, 2009).

Fertilisation: Given that Cobra Lily is carnivorous plant the additional fertilization is unnecessary (Švarc, 2009).

Possible problems with cultivation: The biggest complication is to assure all the necessary conditions for the plant at the same time especially in different times of the year.

Pests and diseases: In home conditions three major problems might occur. These are fungi *Botrytis cinerea* attacking both seedlings and adult plants, putrefaction of rhizomes or growing and developing pitches and aphids attacking adult plants and deforming the pitches.

2.10 Propagation *in vitro* by direct morphogenesis: Carnivorous plants and use of i*n vitro* technologies in carnivorous plants

Some plant might have various barrier in their generative propagation cycle such as low germination rate in case of *D. californica* or the goal is just to get high amount of viable plants in short term which would not be sustainable in case of natural propagation. Thus *in vitro* methods are often implemented.

Micropropagation or plant propagation via tissue culture has a goal to propagate plants true-to-type, called also clones. Three basic ways how to achieve this goal could be:

- Micropropagation from pre-existing shoot or primordial buds
- Following shoot morphogenesis (directly only when derived from mother plant)
- Through the formation of somatic embryos (somatic embryogenesis)

Certain species offer the possibility of reliable method for micropropagation by producing adventitious shoots. This advantage can be enhanced by using plant growth regulators (PGRs). Induction of direct morphogenesis depends strongly on a nature of the plant organ from which the explant was derived. In some genera, direct morphogenesis is unknown or very rare (George *et al*., 2008)

The biggest advantage in this *in vitro* propagation technique is avoiding step with unorganised cells, so called callus (George *et al*, 2008; Bhojwani *et al.*, 1996). Callus stage is arise the risk of unwanted genetic changes thus regeneration of plants with a different genetic identity.

Several ornamental plants are at present propagated *in vitro* by direct shoot regeneration. Chief among these are plants of the family Gesneriaceae, (including

Achimenes, *Saintpaulia*, *Sinningia* and *Streptocarpus*), where shoot buds can be freely regenerated directly on leaf explants without the formation of any intervening callus phase. Many other ornamentals and crop plants either are (or could be) propagated efficiently by this means, for example, begonias, *Epiphyllum*, cacti, *Gerbera*, *Hosta* and *Lilium* (Bhojwani *et al.*, 1996).

In the case of *Darlingtonia*, micropropagation via direct morphogenesis could be convenient for propagation given the fact that germination rate of its seeds is decreasing rapidly with time. Despite the small commercial production *of Darlingtonia*, any official optimised protocol for its micropropagation has not been found.

However some works dealing with *in vitro* propagation of carnivorous plants exist, namely for plants from family Droseraceae-sundews.

An example is a protocol for *in vitro* propagation of *Drosera intermedia* (Grevenstuk *et al.*, 2010) where they developed simple yet efficient protocol using MS medium of 1/4 salts concentration and 2% sucrose. Their culture were established from seed despite that the *Drosera* species are more commonly started by leaf segments or shoot tips (Bobák *et al*., 1995; Kawiak *et al.,* 2003). They also obtained high rate of rooting on a medium without PGR and according to this study, addition of 0.1 $mg{\cdot}l^{-1}$ of kinetin had no significant effect on multiplication of this species.

Another study dealing with *Drosera* species was performed by Anthony J. (1992) who developed protocols for *D. rotundifolia, capensis* and *binata*. He tested MS medium with 1/2, 1/4, and 1/8 salt concentration but 3% of sucrose with full MS as a control. He used concentration of 0.02 $mg{\cdot}l^{-1}$ BA and 0.01 $mg{\cdot}l^{-1}$ NAA for proliferation and 1/2 PGR free MS for rooting. He obtained best results for 1/4 and 1/2 MS. He also mentions problems with contaminations posing a question of symbiotic microorganisms that could remain in intercellular spaces in cultured plants and may occasionally be stimulated to resume growth.

By shoot tip culture was propagated another species of *Drosera* family, *D. peltata*, an endemic tuberous sundew from Bokil island, South Korea (Kwang-Soo and Gi-Won, 2004). They tested four media: MS, B5 (Gamborg *et al*., 1968), LS (Linsmainer and Skoog, 1965) and RM (Reinert and Mohr, 1967) and for evaluation of the medium concentration effect, five MS concentrations were tested (2 MS, full, 1/2, 1/4 and 1/8) and the effect of cytokinins was evaluated on 1/2 MS supplemented with kinetin or benzyladenine. They also tested

effect of medium pH. Interestingly, explants placed on 2 MS all died, optimal pH found was 5.7 and with severe inhibition in low pH. They obtained best results on 1/2 MS without PGR.

In ancient carnivorous species *Cephalotus follicularis* from monotypic family Cephalotaceae from Australia even lower concentration of mineral salts has been proved to be best (concentration 1/5 and 1/10 MS without PGR) for shoot proliferation and development. The use of liquid LS medium was also proved to be highly effective (Chia-Yun *et al*., 2010).

For probably the most famous carnivorous plants, Venus fly trap (*Dionaea muscipulata* Ellis) few method of micropropagation has been described (Beebe, 1980; Parliman *et al.,* 1982; Minocha 1985; Hutchison, 1984; Teng, 1999). Gi-Won *et al.* (2003) developed a protocol for micropropagation of venus fly trap by shoot culture by optimization of medium, pH and PGR for both shoot proliferation and root formation. They found the best shoot proliferation on 1/3 MS. Following these results, they tested concentration of auxins (kinetin and benzyladenin) on 1/3 MS and found out that BAP supplementation suppressed shoot proliferation. They had the best results for 1/3 MS supplemented with 0.5 kinetin $mg{\cdot}l^{-1}$. For rooting 1/3 MS supplemented with 0.1 $mg{\cdot}l^{-1}$ IBA gave the best results. On the other hand, additions of 2, 4-D or NAA strongly suppressed root formation and without PGR root formation achieved 70.8%.

Some other species of carnivorous plants that are regarded as medicinal plants were propagated *in vitro* for performance of various bioassays mainly for production of secondary metabolites. For example Hook (2001) tested *in vitro* cultured carnivorous plants for content of naphthoquinones. *Dionaea muscipulata* on PGR free medium produce 5.3% of plumbagin but *Drosera binata* only 1.4%. *D. capensis* and *rotundifolia* were tested for 7-methyljuglone. Banasiuk *et al.* (2012) tested *Drosera ramentaceae, D. anglica, D. binata, D. cuneifolia* and *D. capensis* also for the production of biologically active secondary metabolites. More interestingly, they tested six different cultivation mediums (Fast, 1981), Vacin and Went (1949), 1/2 MS, MS, Reinert and Mohr (1967) and Lindemann (Lindeman *et al.,* 1970) with best results obtained always on 1/2 MS.

However, so far there is no known official suitable protocol for micropropagation of *D. californica*.

2.11 Evaluation of genetic stability of regenerants using molecular markers

The direct morphogenesis is based on the assumption that the material is propagated, treated and stored under conditions ensuring genetic stability. However, there are various factors linked with *in vitro* culture procedures that can be a source of variation. There are different factors but also different methods how to detect it by diverse approaches for assessing the genetic stability of plants recovered from *in vitro* culture (Callow *et al.*, 1997).

Harding (1995) reviewed factors of genetic variability and various approaches for its assessing in *in vitro* cultures. He mentions among most important reasons for variability pre-existing genetic variation in the collections, possibly linked to genetic structure and inclusion of a dedifferentiated phase or important stress phase in the culture. Also, the risks of losing the genetic integrity of the plant material through somaclonal variation increase with time in culture (Scowcroft, 1984).

The true-to-type propagation is especially important in commercial propagation and commercial *in vitro* laboratories. According to George *et al.* (2008) there are many cases where nurseries have refused to purchase any more micropropagated plants because of unacceptable somaclonal variationmeaning there is an important reason why to test the regerenants in randomly selected samples.

The fastest and most reliable way how to assess the genetic stability is use of molecular markers, which provide quick and reasonably costly method (Kalia *et al.,* 2011). He reviewed microsatellite markers, their use and recent progress in plants. Molecular markers provide powerful tool and many application including examination of genetic relationships, genetic mapping, constructing of linkage maps, marker assisted selection and backcrosses or population genetics and phylogenetic studies. The general features of DNA markers are summarized in table 1.

Table 1 Selected important features of most common types of molecular markers (Kalia *et al.*, 2011)

Features	Molecular markers			
	EST-SSRs	SSRs	RFLPs	RAPD/AFLP /ISSR
Need for sequence data	Essential	Essential	Not required	Not required
Level of polymorphism	Low	High	Low	Low-moderate
Dominance	Co-dominant	Co-dominant	Co-dominant	Dominant
Interspecific transferability	High	Low-moderate	Moderate-high	Low-moderate
Utility in MAS	High	High	Moderate	Low-moderate
Cost and labour involved in generation	Low	High	High	Low-moderate

In most of the studies where they employed molecular markers for assessment of genetic stability AFLP, RAPD, ISSR or combination of them at the same time was used. AFLP and ISSR markers are often more reliable than RAPD markers, presumably because the former methods employ more stringent PCR conditions. Microsatellite markers are generally claimed to be quite reproducible. Moreover, due to the locus specificity of these markers and the few, small-sized bands exhibited in a single amplification, problems caused by, e.g., insufficient DNA quality, usually can be identified early on (Weising *et al.*, 2005) msap.

There does not exist many studies in which molecular markers have been tested on *in vitro* propagated carnivorous plants but Kawian and Łojlkowska, 2004 studied genetic fidelity in micropropagated *Drosera* (*D. anglica* and *D. binata*). They used RAPD markers on 15 samples from each species. They have been regenerated by adventitious budding or shoot tips respectively. They did not detect any polymorphism among *D. binata* whereas *D. anglica* showed 0.08% polymorphism frequency. Their results are proving that the

regeneration through shoot-tip culture is a low-risk method, whereas material regenerated through leaf explants requires further verification.

Other interesting species of carnivorous plant, *Nepenthes khasiana* with medicinal use from India was propagated *in vitro* and clonal fidelity was assessed by Devi *et al.* (2013). They did also cytological analysis to test any possible changes in chromosome number. For testing genetic fidelity, RAPD analysis was performed. From total of 72 amplification products with 4.1% bands showing polymorphism and cluster analysis of RAPD profile revealed an average similarity coefficient ranging from 0.98 to 1.0 suggesting genetic stability.

Very often, an endangered species or species with medicinal use have been micropropagated and evaluated for genetic fidelity. For example *Celastrus paniculatus* Willd., after propagation *in vitro* was showing 100% monomorphism when tested by RAPD and ISSR (Senapati *et al.*, 2013).

So far, *Darlingtonia californica* has not been subject of analysis by molecular markers, neither for horticulture purpose neither for phylegenetic studies. However taxonomical study of Sarraceniaceae including *D. californica* was done (Ellison *et al.*, 2012) but the data for analysis was based on comparison of nuclear, mitochondrial and plastid sequence data. Lacking of other deep studies, elaborated protocol for molecular any markers does not exist. This work is thus aiming for optimising a convenient, feasible protocol and part of this work is assessment of genetic stability for which such a protocol is more than needed.

3. Aims of thesis

This work was aiming to develop an efficient and accurate protocol for rapid micropropagation of *Darlingtonia californica* Torr. through direct morphogenesis and detection of genetic stability/instability of *in vitro* regenerants by means of molecular markers ISSR.

The objectives could be divided into following scheme with partial goals:

- Establishment of a sterile *in vitro* culture of *Darlingtonia californica* from seeds.
- Establishment of a protocol with optimal combination of PGRs in cultivation medium for enhancing direct morphogenesis thus mitigating the risk of somaclonal variability.
- Finding an appropriate protocol for sufficient rooting and transferring the plants into *ex vitro* conditions.
- Evaluation of genetic stability or variability of regenerants from media with best results using inter single sequence repeat markers.

Direct organogenesis is a side track to avoid unwanted somaclonal variation. However the occurrence of genetic changes cannot be omitted and therefore need to be tested to prove true-to-type clones by means of molecular markers and flow cytometry.

4. Materials and methods

4.1. *In vitro* cultures

4.1.1. Establishment of *in vitro* culture

The *in vitro* culture of *Darlingtonia California* has been established during the academic year 2012/13. The plant materials used for establishment of the culture were seeds bought via e-shop.

The protocol used for disinfect the seeds was modified protocol by Illiev *et al.* (2010) (see protocol 1. annex). The disinfected seeds were placed on a fresh 1/2 MS medium (Murashige and Skoog, 1962) in Erlenmeyer flasks (100 ml) and placed to the cultivation box with 16 hours of light and 8 hours of dark per day, at temperature 25/23 °C (light/dark period) and with light intensity of 2000 lx. Other parts of disinfected seeds (approx. 30 seeds) were placed to regular fridge for period of two respectively three months, according to exchange of advices by amateur growers worldwide, then placed to cultivator.

4.1.2. Propagation of plant material for experiments

The plant material for experiment was cultivated and propagated on a basic MS medium (Murashige and Skoog, 1962, see protocol 2 in annex) with half of the salts, supplemented with 15 $g \cdot l^{-1}$ of sucrose and 100 $mg \cdot l^{-1}$ of *myo*-inositol but without any plant growth regulators. The solidifying agent was agar at concentration of 8 $g \cdot l^{-1}$. The pH was adjusted to 5.7 with KOH and acetic acid.

The plants were kept in cultivation chamber with same conditions as for germination (see paragraph above).

With use of this basic 1/2 MS medium, sufficient amount of plants for further experiments material was obtained. Plants were regularly transferred to fresh media of same composition.

All the plants were issued from one single germinated seed thus there were no need to determinate and keep records of the new plants pedigree. The plants were passaged to a new fresh medium of same composition (1/2 MS) regularly within four week. By this mean, sufficient amount of plants needed for experiments were obtained.

However during the cultivation occurred serious problem with diverse contaminations in period of time from October 2013 to end of February 2014. We did all precautions to avoid the spreading of these contaminations (bacteria and fungi) but not successfully in many cases so that numerous cultures had to been discarded. The contamination seemed to be completely eliminated by the end of March 2014.

4.1.3. *In vitro* propagation

The control plants were cultivated on PGR free 1/2 MS medium as described in paragraph above.

Plantlets used for testing PGR supplemented media used were as uniform size as possible. The segment of height approximately 10 to 15 mm and five to seven leaves were used because according to experiences acquired during the cultivation and propagation of plant material for experiments, taller and more developed plantlets had tendencies to browning and senescence their leaves until the eventual death, especially if they were transplanted to new medium in reduces and/or tall rosettes. On the other hand, smaller and more robust rosettes support better its division and adapt faster. Every given row of experiments left 4 weeks to grow before evaluation.

Totally, twelve different varieties of media supplemented with PGRs of different concentrations as described in Table 2 were tested. As a control 1/2 MS medium was used. Each set of medium had been cultivated four week and evaluated for number of shoots after this period of time.

The statistical evaluation was performed using analysis of variance (ANOVA) to determine significance and the significantly means were identified by the Tukey's test ($p=0.05$) [StarSoft STATISTICA 9.0].

Table 2 Varieties of tested experimental medium for in vitro regeneration

PGR 1.	Concentration [mg·l^{-1}]	PGR 2.	Concentration [mg·l^{-1}]
	0.1		
Zeatin	0.5	-	
	1		
	0.1		0.1
Zeatin	0.5	NAA	0.1
	1		0.1
	0.1		
BAP	0.5	-	
	1		
	0.1		0.1
BAP	0.	NAA	0.1
	1		0.1

4.1.4. Rooting

For rooting, six different media were used (Table 3), consisting of one third of MS salts concentration, supplemented with different concentration of naphthalene acetic acid (NAA). One media with half strengthen MS media was also used. For each variety of media, five plants were used.

Table 3 Varieties of tested experimental media for rooting

MS salts concentration	NAA [mg·l^{-1}]
1/2 MS	0.005
	0.1
	0.2
1/3 MS	0.3
	0.4
	0.5

4.2. Molecular analysis

4.2.1. DNA Isolation

The DNA material was isolated from leaf samples of *Darlingtonia californica.* Ten randomly chosen samples from variety with best results and one control from plant cultivated on PGRs free 1/2 MS were analysed. We used method of isolation based on CTAB (Cetyl trimethylammonium bromid) (Doyle and Doyle, 1990; Williams *et al.*, 1992, see annex protocol 2).

The isolation of DNA was done in laboratory of molecular biology of the Department of Crop Sciences and Agroforestry and the quality of isolated DNA was analysed on a Nanodrop Spectrometer (Thermo Scientific, USA) in the laboratory of Interfaculty Centre of Environmentalist Sciences at CULS.

The final concentration of all DNA samples was adjusted according to results of spectrophotometric measurements to 50 $\eta g \cdot \mu l^{-1}$ which is optimal concentration for PCR reactions. DNA samples were stored in -20 °C.

4.2.2. Analysis by ISSR markers and data analysis

A set of 17 ISSR primers (University of British Columbia, UBC, USA) was used for screening. A random isolated DNA from *D. californica* with concentration 50 $\eta g \cdot \mu l^{-1}$ was used to screen suitable primers. Final number of selected primers was twelve as shown in Table 3. ISSR primers that showed high polymorphism were selected for the final analysis (shown in Table 3 with temperatures of annealing).

Each 20 µl PCR reaction mixture was composed of 10 µl of 2x 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 Taq-Purple DNA polymerase, monoclonal antibody anti-Taq (38 nM), stabilisers and additives] (Tob-Bio, Czech Republic) 0,5 µl of respective ISSR primer (Integrated DNA Technologies, Belgium), 2 µl of DNA sample (50 $\eta g \cdot \mu l^{-1}$), 0.2 µl of BSA (Thermo Scientific, USA) and 7.3 µl PCR H_2O (Top-Bio, Czech Republic). The ISSR analysis was carried out using QB96 Server Gradient Thermal Cycler (Quanta Biotech, United Kingdom).

The PCR was carried out with modifications of the annealing temperature to optimize the reaction for individual primers. The cycling conditions were as following:

initial denaturation step at 95ºC for 4 min, followed by 45 cycles of denaturation at 94ºC for 1 min, primer annealing with specific temperature ranging from 45 °C to 58 ºC for 45 s was (first, primers were optimized with gradient temperatures ranging from 45 °C to 58 ºC and primers chosen according to best temperatures of annealing, Table 4), and extension at 72 ºC for 2 min, followed by a final extension at 72 ºC for 10 min. Amplified products did not have to been mixed with loading dye since it is already part of PPP Master Mix. Finally, samples were loaded into slots of the gel. Electrophoretic separation was performed on 2% agarose gel in 1x TBE buffer. Gels were run for about 2.5 – 3 h at 4 $V.cm^{-1}$. Gels were stained with ethidiumbromid (4 µl) and visualized with a UV transilluminator. Banding pattern was recorded using the CSL-MICRODOC System (CLEAVER, United Kingdom).

Table 4 Selected primers and their characterization

No.	Primer code (UBC)	Sequence 5´- 3´	Annealing temperature (°C)
1	'UBC810'	AG AG AG AG AG AG AG AG T	50.7
2	'UBC828'	TG TG TG TG TG TG TG TG A	52.3
3	'UBC834'	AG AG AG AG AG AG AG AG YT	50.7
4	'UBC835'	AG AG AG AG AG AG AG AG YC	54.5
5	'UBC840'	GA GA GA GA GA GA GA GA YT	52.3
6	'UBC841'	GA GA GA GA GA GA GA GA YC	49.3
7	'UBC846'	CA CA CA CA CA CA CA CA RT	52.3
8	'UBC847'	CA CA CA CA CA CA CA CA RC	52.3
9	'UBC851'	GT GT GT GT GT GT GT GT CT G	50.7
10	'UBC854'	TC TC TC TC TC TC TC TC RG	50.7
11	'UBC856'	AC AC AC AC AC AC AC AC YA	52.3
12	'UBC873'	GACA GACA GACA GACA	50.7

4.3. Flow cytometry

DNA-ploidy levels were estimated by flow cytometry using the two-step methodology according to Dolezel *et al.* (2007). *Zea mays*, cv. CE-777, 2C = 5.43 pg (Lysak and Dolezel, 1998) was used as internal standard. Approximately 1 cm^2 from each sample and an appropriate amount of internal standard were chopped with razor blade in 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20). The suspension was filtered through a 42 µm nylon mesh and incubated at room temperature for at least 5 min. Thereafter, it was stained with 1 ml of Otto II buffer (0.4 M $Na_2HPO_4.12H_2O$) supplemented by AT-selective fluorescent dye DAPI (4´,6-diamidino-2-phenylindole) and 2-mercaptoethanol in final concentrations of 4 $\mu g \cdot ml^{-1}$ and 2 $\mu l \cdot ml^{-1}$, respectively. After short incubation (2–3 min) at room temperature, relative fluorescence intensity of at least 3,000 nuclei was recorded using a CyFlow Space flow cytometer (Partec GmbH, Münster, Germany). Data were analysed using the FlowMax software (Partec, GmbH, Münster, Germany). For each analysed sample, DNA ratios were counted by dividing the mean of the G0/G1 peak of the studied plant by the mean of the G0/G1 peak of the internal standard.

5. Results

5.1. Results of micropropagation

5.1.1. Results of *in vitro* propagation

For evaluation of suitability of given medium, we counted number of shoots of every given plant. General appearance of plants was also observed. The results are summarized in the Table 5 together with statistical evaluation.

Table 5 Results of *in vitro* propagation

NAA ($mg \cdot l^{-1}$)	Zeatin ($mg \cdot l^{-1}$)	BAP ($mg \cdot l^{-1}$)	No. of shoots/explant-means±S.D.
-	0.1	-	2.50±0.33 b,c
-	0.5	-	4.20±0.33 a, b
-	1	-	4.20±0.49 a, b
0.1	0.1	-	2.80±0.44 a, b, c
0.1	0.5	-	4.50±0.40 a
0.1	1	-	4.10±0.43 a, b
-	-	0.1	2.90±0.31 a, b, c
-	-	0.5	3.30±0.42 a, b, c
-	-	1	3.77±0.49 a, b, c
0.1	-	0.1	2.75±0.39 a, b, c
0.1	-	0.5	2.80±0.39 a, b, c
0.1	-	1	3.70±0.37 a, b, c
Control	-	-	2.10±0.23 a

Note: In the same column, numbers followed by the same letter are not significantly different (Tukey´s test, $p < 0.05$)

The optimal medium has been chosen according to the number of shoots per plant as well as general appearance. Therefore, varieties of medium where plants exhibited dwarfism or tiny and thick pitcher leaves were excluded and as the best medium for micropropagation, variety producing highest number of shoots was determined as 1/2 MS medium supplemented with 0.5 mg·l^{-1}of zeatin and 0.1 mg·l^{-1}of NAA (Figure 7). The average number of shoots was 4.50±0.40. Also other media with concentration of zeatin 0.5 mg·l^{-1} and higher gave good results with more than 4 shoots per plant in average. However, differences between results obtained from various treatments were not statistically significant. Also, some problems with browning of leaves leading to death of plants occurred (figure 6).

On the other hand, as the worst medium for *in vitro* propagation appeared to be 1/2 MS supplemented with 0.1 mg·l^{-1} zeatin. The average number of shoots for this variety was only 2.50±0.32. Nevertheless, the differences were not statisticaly significant when compared to other media but on the other hand it is still more than a control with 2.10±0.23 shoots per plant. In general, it can be resumed that lower concentrations produced less shoots than higher concentrations of cytokinins.

However, plants grown in the higher concentration of cytokinins (1 mg·l^{-1}) even though that the number of shoots was high, results were not convenient because the shoots exhibited dwarfish growth, often very inter grown so that mother plant was not possible to recognize and number of shoots complicated the determination of number of shoots. Some of the plants exhibited a problem with browning of leaves eventually leading to death of whole rosette or death of mother plants in the cases where enough of viable shoots were already developed.

When 1/2 MS medium with zeatin of above-mentioned concentrations was enriched with NAA at of concentration 0.1 mg·l^{-1} no significant change of number of shoots was observed in comparison to variety without NAA. The plants were of better visual appearance meaning especially the shape of pitchers, which were more regular, slightly taller and of darker green color.

In the 1/2 MS medium supplemented with BAP in same concentrations as for zeatin, the plants formed more shoots in small concentration of BAP (0.1 mg·l^{-1}) than in same concentrations of zeatin. However the plants had distinguished morphology. They were very short in height and in small concentrations of BAP the leaves were very thin and delicate.

Also a problem with browning of leaves appeared suggesting the browning has no relation to variety of medium but rather the size and viability of experimental segment.

Plants cultivated on BAP medium supplemented with of NAA of concentration 0.1 mg·l^{-1} had very similar ratios of shooting and there was no distinguishable difference to variety of 1/2 MS medium with BAP. However the browning occurred even in higher percentage and the plants also had very thin and tiny pitchers. Results suggest that the browning might be caused by small size of rosette of segment used for establishment of culture.

In general, for all PGRs used, higher concentration provoked increased formation of shoots but in the highest concentration used the plants had unnatural appearance and tendencies to grow tiny, thin pitches accumulated together to form dwarfish clustered rosette.

No spontaneous formation of roots appeared in any experimental media during the tests.

None of the plants formed "hoods" on pitches, typical for *Darlingtonia californica.* The formation was observed on few control plants cultivated on 1/2 PGR free MS medium. The segments chose for experiments were of similar age and size with exclusion of mother plant, where recognizable. It is suggesting that the formation is more related to age than composition of medium.

Figure 6 Example of browning of leaves (on 1/2 MS with 0.5 BAP mg·l^{-1}) (source: author)

Figure 7 Example of plant grown on 1/2 MS medium with 0.5 $mg \cdot l^{-1}$ of zeatin and 0.1 $mg \cdot l^{-1}$ of NAA (source: author)

5.1.1. Results of *in vitro* rooting

Unfortunately, any of the tested medium produced *in vitro* rooting thus we could not carry out *ex vitro* transfer and hardening. Neither during propagation experiments, has any spontaneous rooting occurred with longer time of cultivation.

5.2. Results of molecular analysis

5.2.1. Isolation of DNA

Sufficient concentration of isolated DNA was achieved with modified CTAB method (Doyle and Doyle, 1990; Williams *et al.*, 1992). The lowest concentration measured was 123.2 $\eta g \cdot \mu l^{-1}$ and highest was 447.5 $\eta g \cdot \mu l^{-1}$ with average concentration 210.8 $\eta g \cdot \mu l^{-1}$. The absorbance ratio $R_{260/280}$ ration ranged from 1.89 to 2.17. This ration should not exceed 2 (meaning contamination with residues of organic compounds) however DNA close to 2 is still suitable for further use.

5.2.2 Results of ISSR markers

During the optimization of protocol, we selected twelve primers out of seventeen tested that amplified and gave products. Primers no. 'UBC876', 'UBC855', 'UBC845', 'UBC847', 'UBC866' were excluded because they did not give any product of PCR reaction.

In presented thesis, amplified products were obtained by PCR reaction with DNA obtained from eleven plants, ten sample plants from best multiplication medium and one control grown on PGR free MS medium. The donor DNA templates were screened with aim to detect the genetic variability or stability in plants propagated by direct morphogenesis. Out of twelve screened ISSR primers, eleven generated 605 amplified fragments. Out of this amount, none were polymorphic (Table 6, Figure 8). The average number of bands generated by each primer was 5 (range 3 to 8) and the size varied from 400 to 1300 bp. The highest number of bands was generated by primer 'UBC810' and 'UBC834' and the lowest by primer 'UBC856'. The results are summarized in table 6.

Primer 'UBC873' showed unscorable pattern and it could not be included in the results. The sample no. 3 showed in more than one case very weak, badly visible bands suggesting low concentration of DNA. For primers 'UBC851' and 'UBC854' (Figure 9), the

electrophoresis gel visualizing showed that for these two, primers do no sit and anneal at all. However, it would be disputable since very weak bands appeared to be there.

The gel analysis revealed no genetic variability. In sample No. 3, no bands have been obtained, probably due to poor dilution of sample.

Table 6 Scorable ISSR bands amplified by each of 12 selected primers screened to verify stability of plants propagated via direct morphogenesis

Primer code	Primer sequence (5'-3')	Total number of bands amplified	Number of scorable bands per primer (total/polymorphic)	No. and frequency of polymorphic bands per primer	Polymorphic regenerants	Range of amplification (pb)
'UBC810'	$(AG)_8$ T	88	8/0	-	-	500-1100
'UBC828'	$(TG)_8$ A	33	3/0	-	-	1000-1300
'UBC834'	$(AG)_8$YT	88	8/0	-	-	400-1300
'UBC835'	$(AG)_8$YC	55	5/0	-	-	500-1200
'UBC840'	$(GA)_8$YT	55	5/0	-	-	600-1100
'UBC841'	$(GA)_8$YC	77	7/0	-	-	700-1200
'UBC846'	$(CA)_8$RT	77	7/0	-	-	600-1200
'UBC847'	$(CA)_8$RC	77	7/0	-	-	450-1200
'UBC851'	$(GT)_8$ G	60	6/0	-	-	400-1300
'UBC854'	$(TC)_8$RG	50	5/0	-	-	700-1350
'UBC856'	$(AC)_8$YA	33	3/0	-	-	500-1100
'UBC873'	$(GACA)_4$	-	-	-	-	-
Total		**605**	-		-	-

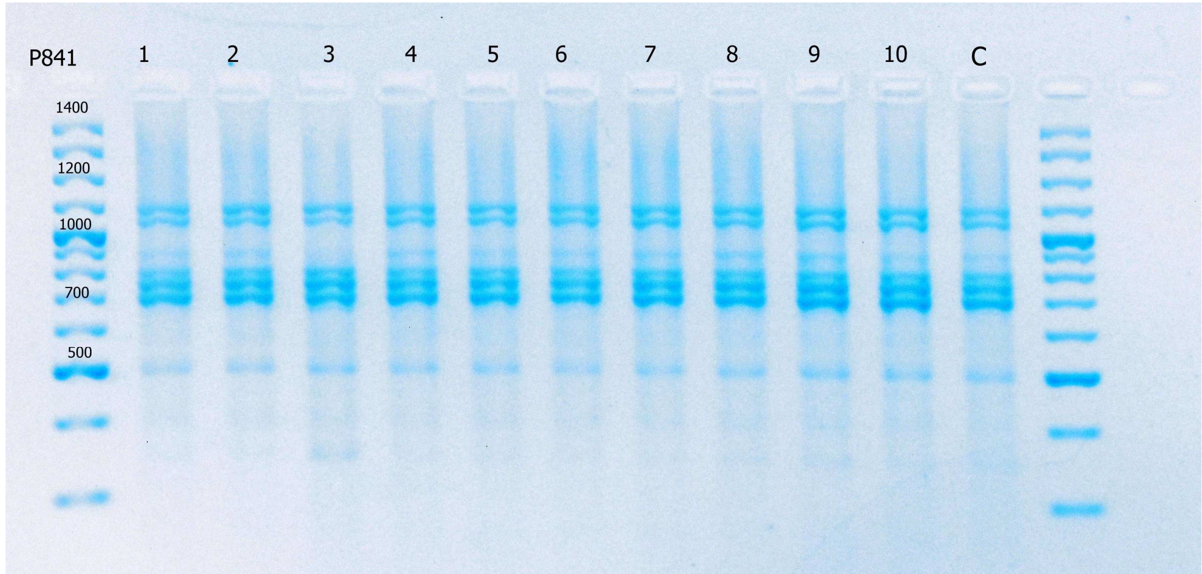

Figure 8 Example of monomorphic pattern screen in primer 'UBC841' with DNA ladder (in base pair) with 'C' being control (source: author)

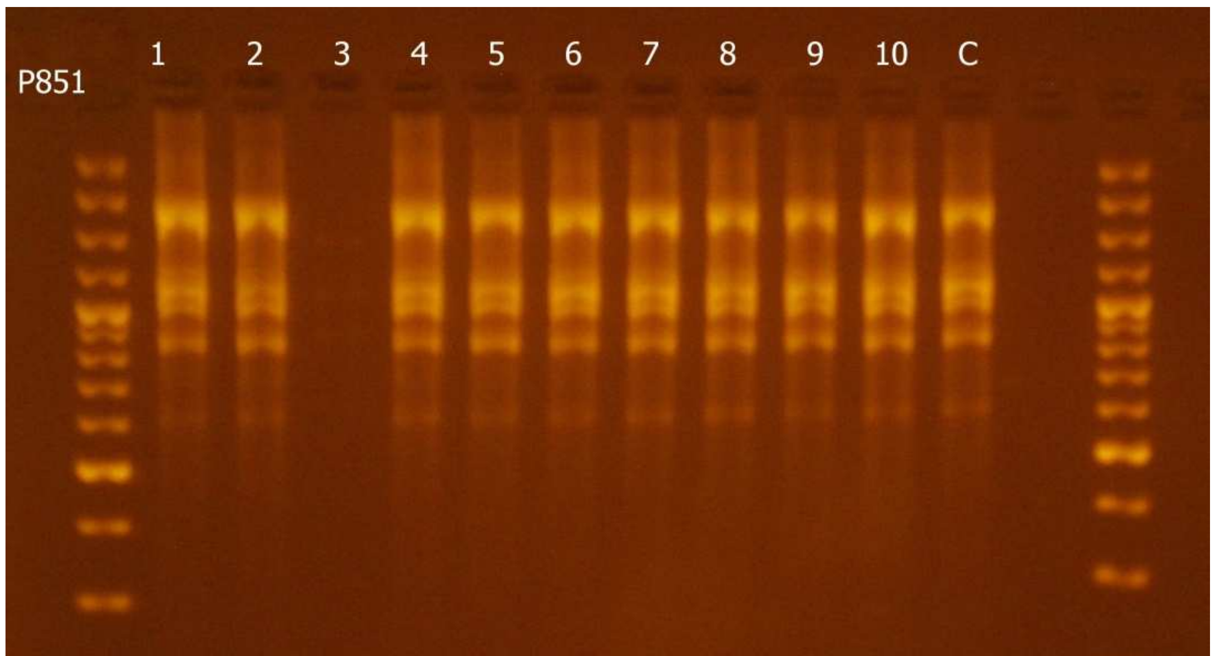

Figure 9 Example of primer ('UBC810'), which does not give product in sample 3, the bands appear but only weak with 'C' being control (source: author)

4.2. Results of flow cytometry

In all cases, linear histograms of relative nuclear DNA content showed two peaks, the first corresponding to somatic nuclei arrested in the G0/G1 phase of the cell cycle, and belonging to the measured sample, and the second representing nuclei of internal standard (*Zea mays*) in the G0/G1 phase (Figure 10 a, b). The DNA-ratios of *in vitro* plantlets varied from 0.347 to 0.353, and they were not significantly different to that of the control plant (0.349), suggesting that the plants after *in vitro* propagation had maintained stable ploidy level. Absence of alterations in ploidy level indicates that there is no somaclonal variation of regenerated plant at the genomic level.

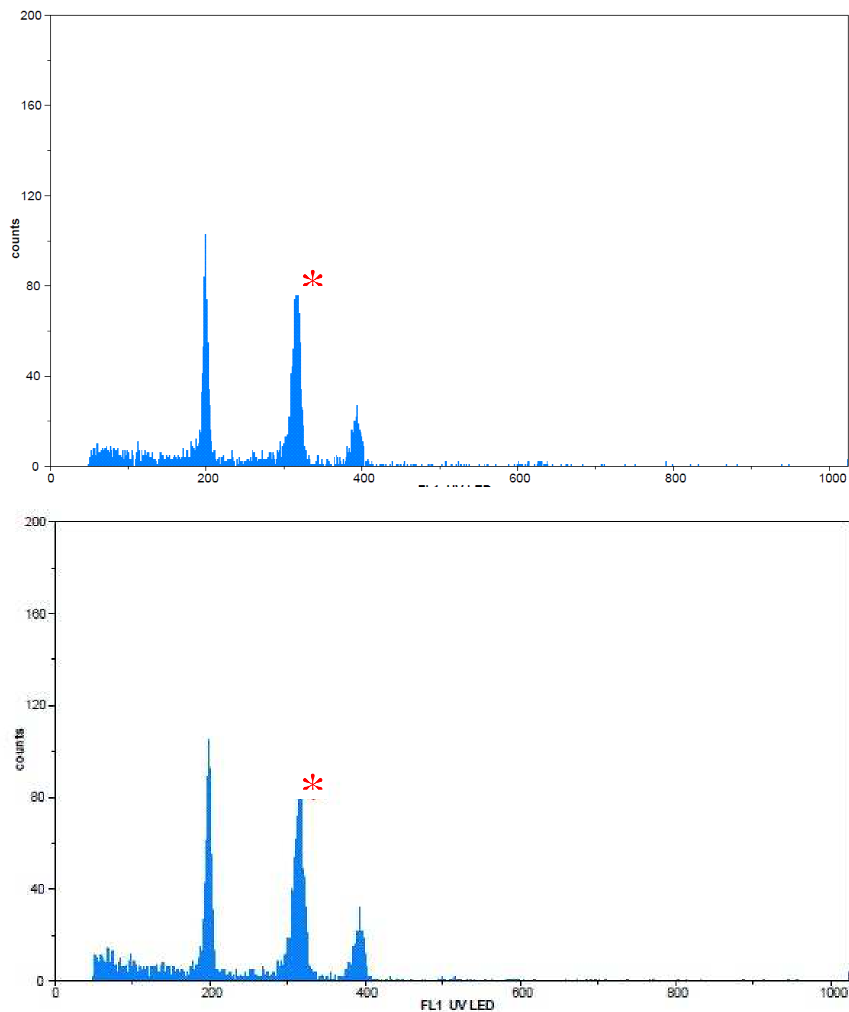

Figure 10 Representative flow cytometry histograms documenting the relative DNA content of in vitro plants. (a) Control plant; (b) randomly selected *in vitro* regenerant. The peak indicated as "*" corresponds to the internal reference standard (*Zea mays*)

6. Discussion

6.1. *In vitro* propagation

Recently, no scientific work dealing with *Darlingtonia californica in vitro* neither with other plants from family Sarraceniaceae does exist; only few reports are available. Most related species to *Darlingtonia*, studied for *in vitro* conservation purpose, were *Sarracenia oreophila* Wherry, *S. leucophylla* Raf. And *S. purpurea* spp. *venosa* Wherry (Northcutt *et al.,* 2012). For these species, media with cytokinins without auxins were optimal. The highest numbers of shoots was produced on BAP, although higher quality of shoots was observed on medium with zeatin. In our study, the best results were provided by medium with zeatin in combination with NAA, even though difference between medium with and without NAA was not statistically significant.

Taxonomically unrelated species, belonging to distinct families such as Droseraceae, Sarraceniaceae or Nephentaceae with different morphology and physiology, however, have one important aspect in common: low nutrient availability of soils where they grow. And, thus, cultivation conditions can be expected to be comparable. There are some studies on *in vitro* propagation of *Drosera* species from family Droseraceae (Bobak *et al.*, 1995; Kawiak and Lojkowska, 2004; Kim *et al.*, 2004; Perica and Berljak, 1996), for *Dionaea muscipula* (Teng, 1999; Jang *et al.*, 2003) from the same family, and for some other carnivorous plants (Chia-Yun *et al.,* 2010; Devi *et al.*, 2013).

For plants from family Droseraceae results of micropropagation seem to be very different from ours. Curkovic and Berljak (1996) concluded that the best propagation medium for *D. spatulata* is medium without any PGRs. Similarly, Kwang-Soo and Gi-Won (2002) reported that the best medium for *D. peltata* is ½ MS medium without PGRs, and Grevenstuk *et al.* (2010) achieved the same result for *Drosera intermedia* and Bobak *et al.* (1995) for *Drosera rotundifolia. Darlingtonia californica* also produce numerous offshoots on ½MS media, however the process is speeded up when PGRs are being employed.

For *Cephalotus follicularis* Labill. from family Cephalotaceae a protocol with specific concentrations of minerals has been developed (Chia-Youn *et al.*, 2010). The most effective medium for shoot proliferation was liquid MS medium with 1/5 or 1/10 strength of macronutrients and full-strength micronutrients supplemented with 0.87 $mg \cdot l^{-1}$ indole 3-

acetic acid. A liquid medium has not been tested for *Darlingtonia* and might be worthy to test since there is a significant difference in case of *Cephalotus*.

Another popular carnivorous plant in which micropropagation had been already optimized is Venus fly trap (*Dionea muscipula*) from monotypic family *Droseraceae*. Gi-Won *et al.* (2002) recommend as a best protocol for shoot production 1/3 MS medium supplemented with 0.1 $mg \cdot l^{-1}$ IBA that is closer to medium selected as the best for *Darlingtonia*.

Very similar results from different experiments are proposing as a best option lower mineral salts concentrations in growing medium. They are also showing that use of PGRs do not always bring better results than PGR-free medium. However, for *Darlingtonia* cytokinins at low concentrations seems to be beneficial.

6.2. Rooting

According to Bhojwani (1996) the concentrations of salts in medium have dramatic effect on *in vitro* rooting. In some cases where multiplication was induced on full-strength MS medium and later on the salt concentration was reduced to half (Garland, 1981) or a quarter (Skirvin, 1979), rooting was induced. As root inducing growth regulators, auxins either natural (IAA, IBA, PAA) and artificial ones (NAA) are being used (George, 2008, Bhojwani, 1996; Kyte, 1996). For some species, so called "auxin synergist" or "rooting co-factors", additional compounds further enhancing the rooting has been tested such as riboflavin (Jarvis, 1986) or aromatic amino acids (Smith, 1997). Such compounds have not been used in performed experiments and despite our continuous attempts none of chosen concentration of auxins brought satisfactory results.

In our study auxins were used in combination of low nutrients concentration without achievement of rooting. Due to results, above-mentioned supplements of rooting medium are to be considered.

For rooting of *Drosera intermedia* very high rooting percentages (up to 100 %) were obtained in multiplication phase on 1⁄4 MS medium without growth regulators (Grevenstuk *et al*., 2010). For *Dionaea muscipulata*, the best conditions for rooting were 1/3 MS medium supplemented with 0.1 $mg \cdot l^{-1}$ (Jang *et al.,* 2002).

Results of these studies are suggesting that low salts concentration and addition of auxins is boosting vigorous rooting. Nevertheless, in our study, despite very low

concentrations of MS nutrients and sucrose in medium, no effect on *in vitro* rooting was achieved.

According to Boulay (1995) two major problems with rooting of *Darlingtonia* are that it does not produce enough roots *in vitro* and thus rooting is not easy to induce and second, once the roots established, they are very sensible to fungi attacks. He proposes to use fungicide treatment and place the young plants in the small greenhouse but we had no opportunity to try the treatment since no rooting occurred. However, his published data are not very specific about protocols.

Kyte and Kleyn (1996) mention activated charcoal as frequent supplement for rooting media to adsorb root-inhibiting agents. That is a possible option worth to try with *Darlingtonia californica* since all the attemps tested during experiments for this master thesis failed.

6.3. DNA isolation

Darlingtonia californica does not produce the sticky mucilage like sundews nor the production of digestives enzymes to the inner cavity of pitcher. It does not contain high amounts of polyphenols, sugars or any secondary metabolites. Therefore, there was no known predisposition for complication with DNA isolation and the chosen method was proved as effective yet time consuming when compare with isolation by commercial kit (Čurn *et al.*, 2008). DNA of *D. californica* isolated by this method was of average quality and good concentration.

There are many other studies for which CTAB method of isolation, or CTAB based method was used. Even for sundew carnivorous plants, protocols based on CTAB are employed (Bekesiova *et al.*, 1999). But this method of DNA/RNA isolation is overall frequently used (Aversano *et al.*, 2011; Linacerero *et al.*, 2011; Borges *et al.*, 2012; Nagori and Purohit 2012; Agbagwa *et al.*, 2012; Schmiderer *et al.*, 2013)

The popularity is most probably thanks to low price compare to commercial kit and can be adjusted to plant species according to the purpose or composition of tissue (Čurn *et al.*, 2008).

6.4. Molecular markers and ISSR analysis of regenerants

Inter simple sequence repeat molecular marker is widely used in regenerants to assess the genetic stability. Recently for example for *Musa banana* 'Williams' (Aremu *et al.,* 2013), *Gerbea jamesonii* Bolus (Bhatia *et al*., 2011), *Aerides vandarum* Reichb.f x *Vanda sangeana* Reichb.f (Kishor and Devi, 2009), *Aloe vera* L. (Rathore *et al*., 2011), *Pongamia pinnata* (L.) Pierre (Kesari *et al*., 2012), *Zingiber rubens* Roxb (Mohanty *et al*., 2011) and many others ISSR markers were used..

Fan et al. (2013) tested in vitro regenerants of *Hylocereus undatus* for somaclonal variation using ISSR. They used 66 primers generating 442 bands revealing no polymorphism indicating high level of genetic stability. Results of ISSR analysis in *Darlingtonia californica in vitro* regenerant revealing genetic stability are supported by their results, showing the direct morphogenesis is producing true-to-type plants.

For carnivorous plants, Kawiak and Łojkowska (2004) used RAPD for determination of genetic stability in clones of micropropagated *Drosera anglica* and *D. binata*. No genetic variation was detected among *D. binata* regenerants but for *D. anglica* a 0.08% polymorphism frequency was estimated. These results revealed that the regeneration of plants through shoot-tip culture is a low-risk method for generating genetic variability.

Nephentes khasiana Hook. was regenerated *in vitro* from nodal segments and the clonal fidelity was also established by RAPD markers (Devi *et al.* 2013). 14 primers resulted in clear and scorable bands, totally 72 amplification products were obtained out of which only 4.1 % bands were polymorphic thus suggesting genetic stability. On the other hand, for same plant, single primer amplification revealed an increase of genetic variation from 4.3% to 10% from the first to the third regenerations (Devi *et al.,* 2014).

Such studies testing clonal stability in micropropagated plants are in accordance wih results of presented master thesis proving that the clonal fidelity in plants propagated *in vitro* by direct morphogenesis is very high an the risk of genetic variability is very low which was proved by using of combination of ISSR and flow cytometry

6.5. Flow cytometry

Flow cytometry revealed no variability in ploidy levels. There is was no somaclonal variation of *Darlingtonia califorica* regenerants suggesting reproducibility of the micropropagation protocol. Flow cytometry is widely used to determine overal genetic variability in regenerated plants. Recently, this method is used, for example, to determine genetic changes in plantlets of *Gentiana pannonica* Scop grown from somatic embryos (Fiuk *et al*., 2010).

Somaclonal variation in cotton (*Gossypium hirsutum)* with use of flow cytometry was tested by Jin *et al*. (2008). The regeneration through callus was reason of aneuploidy of some samples.

Lemon (*Citrus limon* L.) in various tissue culture derived plant populations was tested through flow cytometry by Orbović *et al*. (2008). They tested 240 already acclimated plants, they found one trisomic plant and two chimeras (trisomic buds and diploid roots). According to Pfosser *et al.* (1995) and Lourieiro *et al.* (2005) the flow cytometry reveals only large differences in genome size meaning that low level of aneuploidy could not be totally excluded.

Other examples of use of flow cytometry could be analysis of regenerants of *Spathiphyllum* 'Supreme' (Zhao *et al*., 2012), testing of ploidy stability of adventitious shoots of sour cherry 'Čačanski Rubin' (Vujović *et al.*, 2012) or detection of somaclonal variants in somatic embryogenesis-regenerated plants of *Vitis vinifera* (Prado *et al.*, 2010). These studies did not revealed any changes in ploidy like in presented results of *Darlingtonia*.

Conclusion of all these mentioned studies are suggesting that the somaclonal variability is significantly higher when the regeneration is indirect and that this methodology is more effective when the genome is affected by important big changes.

Because in our study, we induced formation of shoots via direct morphogenesis there was none or very low expectation for change of ploidy. But in experiences of other scientists, such event might occur anyway (Jin *et al*., 2008; Orbović *et al*., 2008).

7. Conclusions

In accordance with presented results, major following conclusions can be stated:

- It was found that the seeds were losing their ability for germination extremely rapidly.
- The optimal medium for *in vitro* propagation was 1/2 MS supplemented with 0.5 $mg{\cdot}l^{-1}$of zeatin and 0.1 $mg{\cdot}l^{-1}$. Use of high concentrations of cytokinins was not reasoned neither from economical point of view nor from the morphological point of view. Plants had strong and negative response to high concentrations of cytokinins. They were forming abnormally dwarfed rosettes.
- The *in vitro* rooting has not been achieved on any of tested media.
- Satisfactory quality of DNA was achieved by isolation with CTAB.
- The plants ploidy was found to be very stable within regenerated plants by using flow cytometry.
- Regenerated plants were genetically stable as confirmed by ISSR analysis.

According to the results discussed and summarized in previous chapters it can be concluded that the main objectives of the diploma thesis were fulfilled. However a partial goal focused on ex vitro transfer could not be achieved, due to insufficient root formation. Thus, more experiments are needed to optimize this phase.

8. Recommendations

Following suggestions and recommendations, based on laboratory experiments and their results, could be pointed out:

- It is recommended to use fresh seeds from proved source for establishment of *in vitro* culture.
- For rooting, an alternative to tested media, 1/2 MS low in sucrose and enriched by active charcoal should be tested since no rooting has been achieved. Low concentrations of auxins should be also tested for rooting, as well as various combinations of auxins.
- For rooting, an alternative liquid medium is also proposed to test since *Darlingtonia* grows in flooded soils and the differences in solidity were important during micropropagation for some species.
- Thorough the standardization of segments size for establishment of experiments is needed. *Darlingtonia californica* grows in flocked rosettes, and thus, it is not always possible to determine the mother plant and shoots. Each segment could have identical predispositions for shooting. Furthermore, segments should have at least six pitcher leaves otherwise they have tendencies to brown that eventually lead to death.
- Test the same selected primers on samples, which did not give any PCR product or revealed unsatisfactory pattern.

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10. Annex

Figure 11 Detail of flowers of *Darlingtonia califonica* (source: Srba, 2013)

Figure 12 Detail of matured flower and seeds (source: Anonymous, terraforums 2006)

Figure 13 Detail of pitcher leaf of *Darlingtonia califonica* (source: Srba, 2013)

Figure 14 Example of *Darlingtonia californica* after three months of growing on 1/2 MS medium (source: author)

Protocol 1. Seed disinfection

(Iliev I. *et al.,* 2010)

Equipment and Reagents:

• Autoclave
• Laminar flow cabinet
• Ultraviolet lamp
• Scalpels, forceps, scissors, glass beakers (100 ml), glass Petri dishes, filter paper, aluminium foil, tweezer, glass beakers (100 ml–1 l in volume).
• Burner
• Distilled water
• Tween 20 (Sigma) (replaced by Jar, kitchen detergent)
• Ethanol: 96% and 70% (v/v)
• NaClO (used commercial product "SAVO")
• Sterile culture medium MS

Method:

1. Prepare disinfect material for working (petri dishes, glass beakers, scalpels, tweezer wrapped in aluminium foil)
2. Disinfect the laminar flow cabinet by exposing the work bench to ultraviolet illumination, spray the work surface of the cabinet with 96% (v/v) ethanol
3. Wrap the seeds into small "pockets" made of filter paper
4. Wash the pockets under running tap water for 5 min.
5. Wash hands thoroughly with soap before working, next steps in flow box
6. Wash the explants (by stirring on magnetic mini-stirrer) in 70% (v/v) ethanol (2 min) and 5% (w/v) NaClO, containing 20 drops per litre of Tween 20 (10 mins). After immersion in each solution, wash the explants 3 times with sterile distilled water
7. Place the seeds on the culture medium (using disinfected tools over burner)

Protocol 2. CTAB method for plant DNA isolation (Williams *et al.* 1992), modified

Chemicals:

ddH$_2$O
Isopropanol
5% CTAB
mercaptoethanol
Chloroform-IAA
TE buffer (Tris/EDTA buffer)
Sodium acetate
70% ethanol and 96% ethanol

Procedure:

- Around 100 mg of fresh plant material is homogenized. We added small amount of sterilized brash, frosted the samples and crushed within 15 s in a breaker.
- 500 µl of solution of 2x CTAB with 1% mercaptoethanol is added (here corresponding to 495 µl CTAB and 5 µl of marcaptoenthanol). Solution is homogenized by vortexing.
- Incubation of samples in 65 °C during 45 minutes. Every 15 minutes, samples are slightly stirred.
- Samples are centrifuged on maximum rotation speed during 10 minutes in centrifuge.
- Supernatant is pipetted to a new 3.5 ml eppendorf tubes.
- 500 µl of chloroform-IAA is added and tubes are shaken 10 minutes.
- Samples are centrifuged on maximum rotation speed during 5 minutess .
- Water phase is carefully pipetted to a new 3.5 ml eppendorf tubes.
- 1/5 of 5% CTAB (here correspond to approximately 100 µl) is added and mixed by vortexing.
- Again, 500 µl of chloroform-IAA is added and tubes are shaken 10 minutes.
- Samples are centrifuged on maximum rotation speed during 5 minutes.
- Water phase is carefully pipetted to a new 3.5 ml eppendorf tubes.
- 2/3 of isopropanol are added and two to three times mixed (by turning).

- Samples are left in freezer in -20 °C 2 hour or whole night.
- Samples are centrifuged on maximum rotation speed during 10 minutes in 4 °C.
- The supernatant is discarded and the sediment is dried.
- 300 µl of 1xTE is added to dissolve the samples (shaking 30-60 min, 37 °C).
- 20 µl of 3M sodium acetate and 600 µl of 100% (96%) cold ethanol are added and two to three times mixed (by turning).
- Samples are left in -20 °C for 20 minutes to 12 h.
- Samples are centrifuged on maximum rotation speed during 10 minutes in 4 °C.
- The supernatant is discarded and the sediment is dried.
- 400-1,000 µl of 70% cold ethanol is added and two to three times mixed (by turning).
- Samples are centrifuged on maximum rotation speed during 10 minutes in 4 °C.
- The supernatant is discarded.
- 400-1,000 µl of 70% cold ethanol is added and two to three times mixed (by turning).
- Samples are centrifuged on maximum rotation speed during 10 minutes in 4 °C.
- The supernatant is discarded and the sediment is dried.
- The sedimented DNA is dissolved in 50-100 µl of H_2O and can be stored in fridge for short term or in freezer for long term.