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In vitro propagation of *Puya berteroniana* and assessment of genetic stability in regenerants using molecular markers

Master thesis

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Certification

I confirm, that this master thesis "*In vitro* propagation of *Puya berteroniana* and assessment of genetic stability using molecular markers" is original result of my work, which I declare by my signature, that this master thesis was written independently by using of cited resources at the end of this work.

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Petra Štréblová

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List of Abbreviations

2,4 –D	2,4-dichlorophenoxyacetic acid
ABA	Abscisic acid
AFLP	Amplified fragment lenght polymorphism
BAP	6-benzylaminopurine
CAPS	Cleaved amplified polymorphic sequence
CTAB	Cetyltrimethyl ammonium bromide
CULS	Czech University of Life Sciences in Prague
DAPI	4´,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization
IAA	Indole-3-acetic acid
IBA	Indole butrytic acid
ISSR	Inter simple sequence repeat
IUCN	Union for Conserving of Nature and Natural Resources
KIN	Kinetin
MAS	Marker-assisted selection
MS	Murashige and Skoog medium (1962)
NAA	α – naphthaleneacetic acid
NT	Near threated species
PCR	Polymerase chain reaction
PGR	Plant growth regulator
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
SRAP	Sequence related amplified polymorphism
SSRs	Simple sequence repeat
TDZ	Thidiazuron
UBC	University of British Columbia
USA	Unites States of America
USDA	United States Departement of Agriculture
ZEA	Zeatin

Abstract

Puya berteroniana (Bromeliaceae) is a plant with very attractive turquoise flowers that have a great potential to be used for ornamental purposes in a large scale. The aim of this thesis was optimization of *in vitro* propagation and assessment of genetic fidelity using molecular markers and flow cytometry. An efficient protocol for *in vitro* propagation via direct morphogenesis was established. Benzylaminopurine (BAP) and zeatin alone or in combination with α – naphthaleneacetic acid (NAA) were examined for their effects on *in vitro* offsets induction. Media containing NAA or indole-3-acetic acid (IAA) did used to optimize rooting. For *in vitro* propagation, the most effective was medium enriched by 0.1 mg.l^{-1} BAP and for rooting medium containing 0.3 mg.l^{-1} NAA. Genetic stability of the regenerated plants was determined by inter simple sequence repeat (ISSR) markers and flow cytometry analysis. Out of 170 plantlets, 10 were used for screening. 11 ISSR primers produced totally 19 clear and reproducible bands resulting in a total of 253 bands where the banding pattern per each primer was highly uniform and identical to the mother plant from which the tissue cultures had been established. Flow cytometric analysis did not reveal differences in ploidy levels. The occurrence of somaclonal variation did not detected and thus, process of *in vitro* production can be used for mass production of *P. berteroniana*.

Keywords: direct morphogenesis · flow cytometry · genetic stability · molecular markers· plant growth regulators · *Puya berteroniana*

Abstrakt

Puya berteroniana (Bromeliaceae) je rostlina s atraktivními tyrkysovými květy, využitelnými pro okrasné účely. Cílem této práce byla optimalizace procesu *in vitro* množení této rostliny a ověření genetické stability za použití molekulárních markerů. Tvorba adventivních odnoží byla testována na kultivačních médiích s přídavkem buď pouze 6-benzylaminopurinu (BAP) či zeatinu, nebo v kombinaci s kys. naftyloctovou (NAA). Pro zakořeňování odnoží byla testována média s přídavky NAA nebo kys. indolyl-3-octové (IAA). Nejefektivnějšího odnožování rostlin bylo dosaženo na médiu s 0.1 mg.l^{-1} BAP a kořeny se nejlépe tvořily na médiu s 0.3 mg.l^{-1} NAA. Genetická stabilita regenerovaných rostlin byla ověřována pomocí ISSR (inter simple sequence repeat) markerů a průtokové cytometrie. Ze 170 rostlin bylo náhodně vybráno 10 regenerantů. 11 ISSR primerů generovalo celkem 253 jasně a dobře reprodukovatelných bandů u vybraných regenerantů. Získaný počet bandů na jeden primer byl 19. Získané spektrum pro daný primer bylo vždy uniformní pro všechny testované vzorky a bylo identické se spekterem mateřské rostliny, která byla použita jako výchozí rostlinný material pro tkáňové kultury.

Klíčová slova: genetická stabilita · molekulární markery · průtoková cytometrie · přímá morfogeneze · *Puya berteroniana* · růstové regulátory rostlin

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1. Introduction

Blue *Puya* [*Puya berteroniana* (Mez)] from the Bromeliaceae family is a perennial plant cultivated mainly in the Chilean region for thousands of years. Plants are harvested by local people mainly for ornamental purposes. This species has a great potential to be used also commercially in a larger scale. However, optimization of mass propagation of plant material is needed.

Recently, *P. berteroniana* is being propagated mainly by seeds. However, sexual way of reproduction is slower and more difficult process than asexual propagation. Development of plants obtained from offsets is more rapid, but propagation coefficient of vegetative propagation is very low. Thus, *in vitro* technologies represent an effective method for large-scale propagation of plants. Direct morphogenesis tends to provide genetically identical plants, necessary for flower mass production. Nevertheless, true-to typness is necessary to asses and exclude the occurrence of somaclonal variation in regenerated plants.

Therefore, the objective of this study was to develop an appropriate method, which could allow the regeneration of plants of *Puya berteroniana* via adventitious shoot and evaluation of genetic stability of *in vitro* regenerants using ISSR markers and flow cytometry.

2. Literature review

2.1. Bromeliads

The Bromeliaceae is a monocot family contains a unique and the fascinating plants, occur mainly in the world tropics and West Indes. Except one species, that occurs in western Africa *Pitcairnia feliciana* (Givnish *et al.,* 2004). Family contains over 3000 described plant in more than 56 genera, that are anatomical, morphological and ecological variable (Mez, 1896; Padilla, 1973; Rauh, 1979). Epyphitic bromeliads are very important for nutritient cycle and supporting of animal communities in the canopy (Lowman and Ganesh, 2013). In general, Bromeliads are inexpensive, it is easily to grown and the requirements for care are very small. The Bromeliads are mainly cultivated for their fascinating long lasting blooms and ornamental foliage. There is a wide range of ornamental plants from miniatury to giant size (Benzing, 2000). Disjunct distribution is a result of recent long range dispersal event (Givnish *et al.,* 2004). Harvesting of bromeliads is common in Neotropics (Haeckel, 2008) especially for ornamental purposes and religious decorations (Lowman and Ganesh, 2013).

Bromeliads were discovered before 500 years, by Christopher Columbus, whose was introduced the pineapple (*Ananas comosus*) to Spain during his second voyage to the New World. Bromeliads needed some time for adaptation and addition into the cultivation system. This period was until 1776, when the other species was introduced to Europe (*Guzmania lingulata*), followed by (*Vriesea spledens*) in 1840 (Benzing, 2000).

The systematic, history and functional biology of Bromeliaceae allow to reconstruct the adaptive radiation and to impute the conditions of wild ancestors and habitats. Structure of DNA provides the robust phylogeny within the family and phenotype is responsible for current adaptive variety in each ecosystem (Benzing, 2000).

During the last hundred years, Bromeliads become more popular and widely used as a ornamental plant around the world, Bromeliads can found in greenhouses, gardens and also in the botanical gardens and in wild. Commercial production began during numerous Old World sites (Benzing, 2000).

2.2. *Puya berteroniana*

2.2.1. Taxonomy and relative species

Puya includes [*Puya berteroniana*] is a large genus of ornamental, terrestrial bromeliads belongs to the Bromeliaceae or Pineapple family and to the Pitcarnioideæ subfamily. Very often is called a „sister" of the Bromelioideæ subfamily (Givnish *et al.,* 2007, Givnish *et al.,* 2004; Horres *et al*., 2000; Terry *et al.,* 1997). Subfamily Pitcarnioideæ is characterized by ovary, fruit and seed characteristics (Harms, 1930; Smith and Downs, 1974; Smith and Till, 1998). According to Givnish *et al.* (2007) the subfamiliar concept is divided into six main monophyletic groups: Brocchinioideae, Linamanioideae, Navioideae, Hechtioideae, Pitcarnoideae and Puyoideae.

The genus *Puya* was firstly recognized by Ignacio Molina in his "Saggio sulla storia naturale del Chili" in 1782. Since the first description of the genus, every year a knowledge about *Puya* incessantly increases (Mez, 1896; Smith and Downs, 1974; Smith and Till, 1998; Luther, 2010).

According to Luther (2004) the genus *Puya* describes more than 200 species. Georaphically isolated group of *Puya* species, which is restricted to the Chilean region (Smith and Downs, 1974) represent a strange centre of diversity within a genus (Varadarajan, 1990).

The genus was divided into two subgenera: *Puya* and *Puyopsis* (Smith, 1970). There is one very important difference between these subgenera. *Puya* shows the fertile flowers along the branches of inflorescence and *Puyopsis* does not show fertile flowers of inflorescence (Smith, 1970; Smith and Downs, 1974). As shown in Figure 1, subgenus *Puyopsis* comprises more species than *Puya*, because according Smith and Downs (1974) the subgenus *Puya* comprises only eight species: *Puya boliviensis, P. raimondii, P. weddeliana, P. berteroniana, P. alpestris, P. chilensis, P. gilmartiniae* and *P. castellanosii.* While the subgenus *Puyopsis* includes 20 species : *P. laxa, P. westii, P. ferruginea, P. nitida, P. floccosa, P. aequatorialis, P. spathacea, P. coerulea, P. venusta, P. medica, P. venezuelana, P. pygmaea, P. aristequietae, P. santossi, P. cuatrecacasii, P. trianae, P. ferreyrae, P. nutants, P. dougotiana* and *P. retrorsa.*

Puya represent an important part of phylogenetic tree from family Bromeliaceae (Schulte and Zizka, 2008).
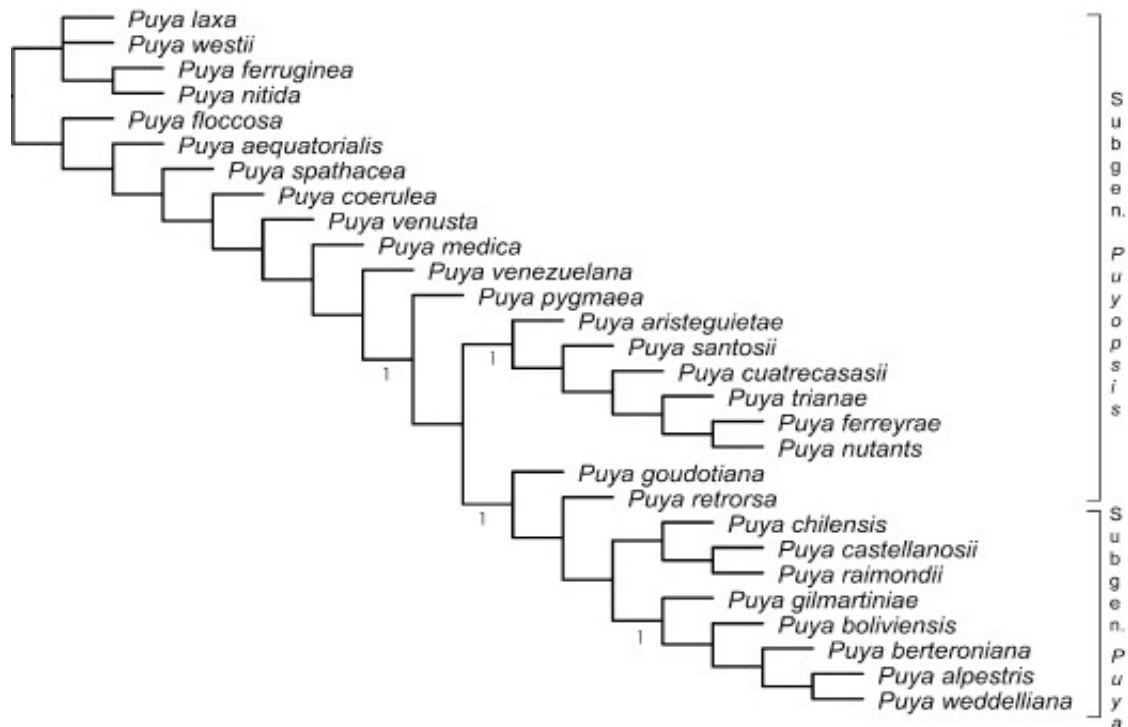
Figure 1. Division of species into subgenera *Puya* and *Puyopsis* according to Hornung-Leoni and Sosa (2007).

2.2.2. Common names

Puya berteroniana is also known as *Pitcairnia berteroniana*. In Great Britain and Ireland is used name Blue *Puya* (Weiliang, 1997) or Turquoise *Puya*. In Chile are used these vernacular names in Spanish language: Chagual also is used Chagualillo, Cárdon (Gourlay, 1952), Maquey, Chagnalillo and Mapache is used in Costa Rica (Benzing, 2000).

2.2.3. Origin and geographical distribution

Plants from genus *Puya* come from the New World, native to southern parts of South America. According to Jabaily and Sytsma (2012), *Puya* species are primarily found from sea level > 4,500 m elevation, but majority of the species we can find above 1,500 m a.s.l., this genus can occur in dry and also in moist habitats. Therefore we can find *Puya* species from Cordillera to Tucumán in Argentina (Jabaily and Sytsma, 2012) and trought Costa Rica (Schulte *et al.,* 2010). Most of *Puya* species are located along to the range of Central America mountains and trough mid and high elevations of Andean mountain and also we can find the occurrence in low elevations of Chile. Mediterranean region represent the lowest elevation range of *Puya* (Jabaily and Sytsma, 2012). This region is separated from the distribution of other *Puya* species in parts of Andes (Jabaily and Systsma, 2010; Rundel and Dillon, 1998; Smith and Downs, 1974). Majority of Chilean *Puya* species represent a very large area of distribution, where the plants occur along climatic and ecological gradient, according this distribution the Chilean *Puya* creates the large morphological variation between species (Schulte *et al.,* 2010).

Puya species are one of the most important recognized components of high elevation, páramo, subpáramo, coastal Chilean matorral and puna vegetation (Luteyn, 1999). As shown in Figure 2, the considerable group of seven species (*P. berteroniana, P. alpestris, P. venusta, P. boliviensis, P. chilensis, P. gilmartiniae, P. coeruela*) are primarily found above the sea level in Central Chile. Two common species are found in the páramo regions of Ecuador – *P. clava-berculis* and *P. bamata. P. berteroniana, P. chilensis, P. gilmartiniae* and *P. alpestris* are originated and restricted to higher elevations of central Chile. *P. boliviensis* can be found in north Chile. *P. wedelliana* mainly grow in southern Bolivia, *P. castellanosi* occurs in Argentina and *P. raimodii* occurs in Peru and Bolivia (Jabaily and Systsma, 2012).

The diversity of wet and dry habitats of *Puya* genus is a huge. According to Jabaily and Sytsma (2012) the vegetative and floral diversity of species shows noticeable morphological variations, structure and color of flowers (see Figure 3).
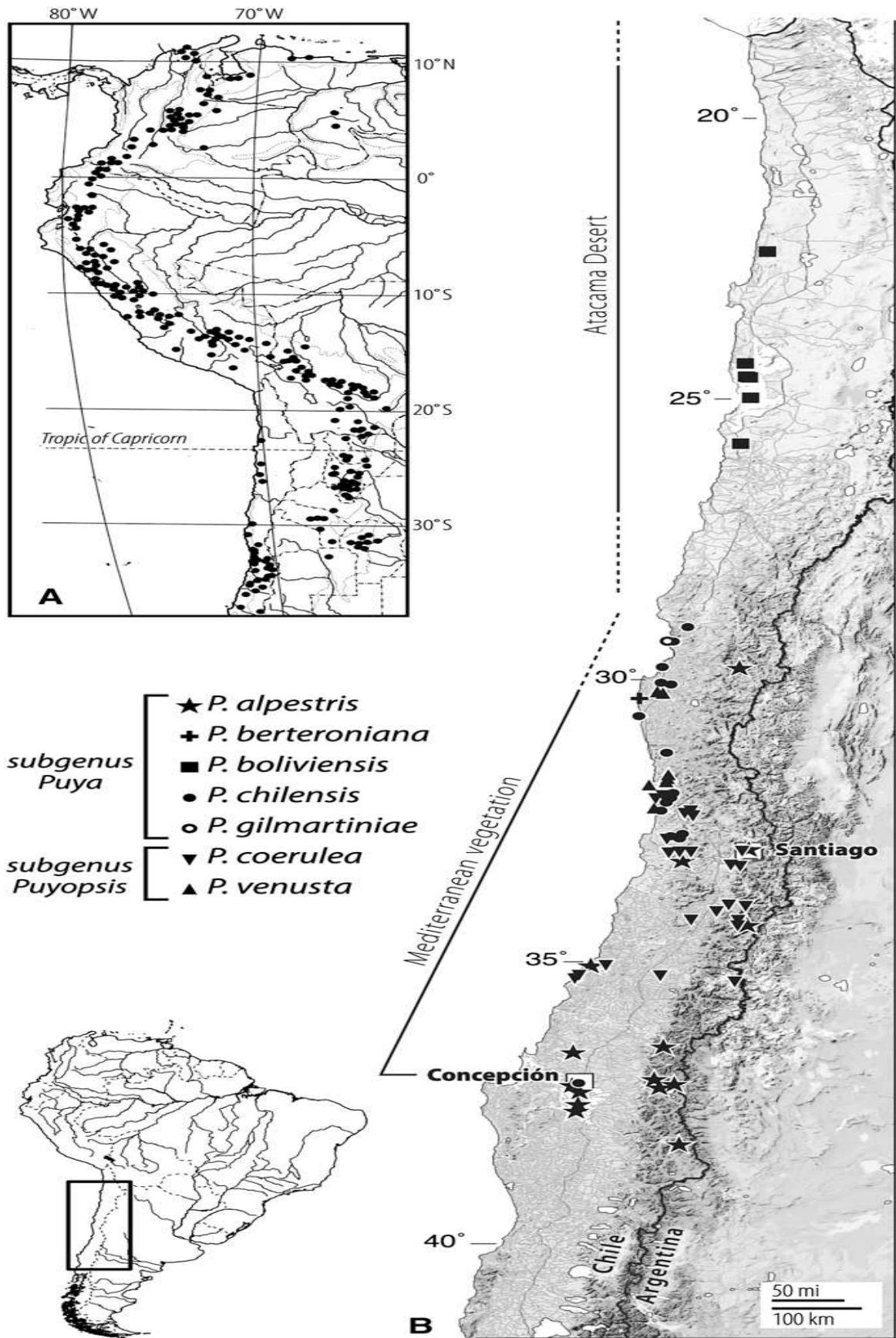
Figure 2. Distribution of *Puya* and *Puyopsis* species based on the herbarium specimen (Rundel and Dillon, 1998; Smith and Downs, 1974).

Figure 3. Diversity of *Puya* species**.** Left gray box shows the members of subgenus *Puya*. Species marked by black line are originated from Chile (Jabaily and Sytsma, 2012).

2.2.4. Ecology

Family Bromealiaceae represents group of plants, which usually grow in grave soil and for their growth, low temperature is typical (Benzing, 2000). *Puya berteroniana* normally requires extremely arid conditions but it is also one of Bromeliaceae plant, that can resist freezing temperatures, but is not tolerant to snow and it is growing in colder climates usually about – 5 °C – typical morning frost of Central Chile (Benzing, 2000). Nowadays these plants are mostly found from sea level close to > 4,500 m elevation (Jabaily and Sytsma, 2012).

Growth of *Puya* is restricted to several environmental conditions. Habitat is divided according to the altitude. *Puya* species can occur in medium altitude up to the timber line and also in low altitude – mainly in valleys and in coastal mountains. As shown in Table 1, *Puya* species can occur in different hardiness zones from 9 to 11 with different range of temperature. Hardiness zones are geographically divided areas according to the temperature and climatic conditions, where the specific category of plant life is capable of growing. These zones in South America include the ability of growing of *P. berteroniana.* This division plays very important role in ability to survive during the minimal temperature of each hardiness zone (Jabaily and Sytsma, 2012).

Table 1. Hardiness zone showing the occurrence of *Puya berteroniana* in different hardiness zones according to, where the small index of letters means the range of temperature in certain region (Jabaily and Sytsma, 2012)

Hardiness zone	Temperature (°C)
9a	to – 6.6
9b	to – 3.8
10a	to - 1.1
10b	to 1.7
11	above 4.5

Puya berteroniana is a drought - tolerant plant but in dry areas, where the drought occurs more than 3-5 months, the requirements of water are about 400–800 mm of precipitations, usually concentrated in winter period.

Dry and arid areas, where the drought period lasting about 6-10 months, requirements of water for plants are about 100–300 mm of precipitations, concentrated in winter period too. Plants require full sun exposure (Benzing, 2000).

Typical kind of soil for *Puya* species is gravel soil. Scale of soil pH is variable from 6.1 to 7.8. It can be grown in mildly acidic, alkaine and neutral soil. It can be also adapted to the loam, loamy sand and sandy loam soils (Benzing, 2000).

2.2.5 Morphology and reproductive biology

Puya genus is effortlessly recognized from others generas by the petal blades, which are diverse from the claw and spiral together after anthesis and by the appendaged seeds, triangular leaf blades and superior ovary (Zizka *et al.,* 2013).

The color of petal is the most important sign for recognition of species group. The groups are divided into the four groups according the color of petals:

1) **Greenish-yellow** - *P. chilensis* group including (*P. boliviensis, P. chilensis, P. gilmartiniae*)
2) **Blue–green** – *P. alpestris* group including (*P. alpestris*)
3) **Blue**- *P. coerulea* group including (*P. coerulea, P. venusta*)
4) **Color is unknown** (Zizka *et al.,* 2013)

From point of view of botanical characteristic *P. berteroniana* was a very problematic species in recognition in region of Chile (Smith and Looser, 1935; Gourlay, 1952), because the identity and taxonomy of this species was not clear (Smith and Looser, 1935). There was no similar species until the study of specimen by professor Benoist (Smith and Looser, 1935) who declared, that the *P. berteroniana* can be synonymous to the species *P. alpestris*.

After this, Smith and Downs (1974) declared according their study that the both species are separate and keep into the *Puya* subgenus. The name *P. berteroniana* is used now for the tropical plant with blue larger flowers with more branches in inflorescence in the comparison with the *P. alpestris* (Zizka *et al.,* 2013).

2.2.6. Habit

Puya berteroniana is a large, evergreen, terrestrial, perennial and rare plant. It usually looks like as agave or grass clump and height is about 3–4.7 m and width is from 0.9 to 2 m. A low stem of plant bears giant rosettes of leaves (Weiliang, 1997).

2.2.7. Foliage

As shown in Figure 4, the simple leaves are spirally and densely arranged into the rosette. The color of leaves is variable from silver to deep gray. The vein is parallel. Base is dilated. The margins are serrate and sharp with visible spines. Spines play important role in plant protection, because the plants use the spines for trapping of anything that can fall into a rosette. *Puyas* are a protocarnivorous plants, they are morphological adaptedto the conditions mainly by spines or trichomes, which is used for traping and killing insect or animals. The animal can trapped and entangled in their spines. Spines are used also for protection from graizing animals. Texture of the leaves is smooth (Zizka *et al.,* 2013).
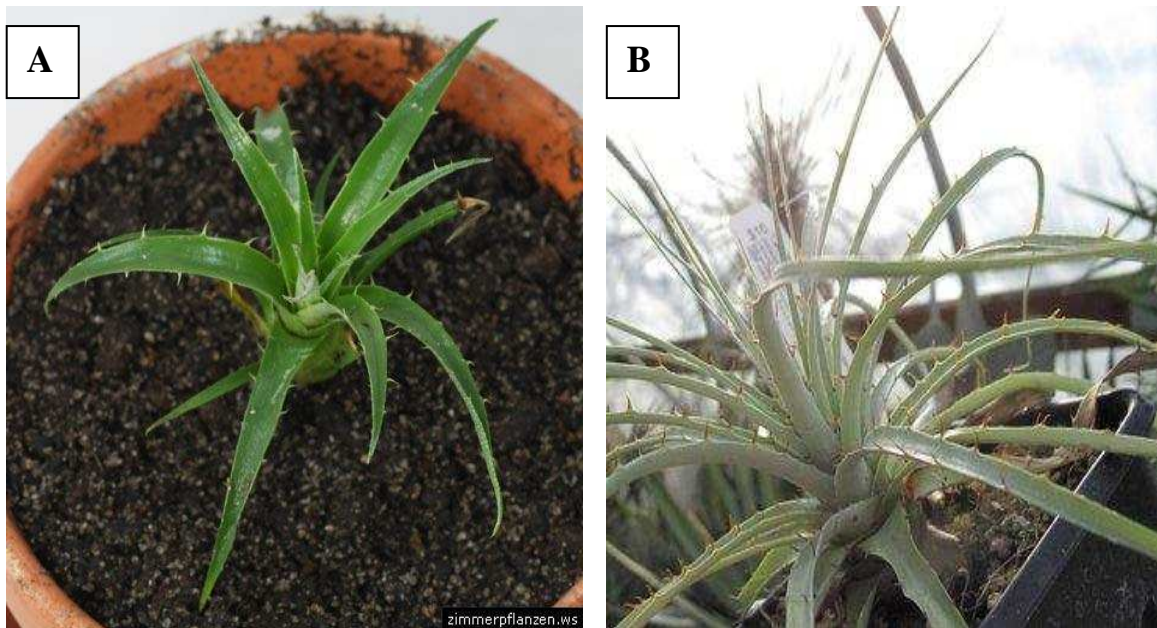
Figure 4. Foliage of *P. berteroniana*. A – Arrangement of leaves into the rosettes, B – spines on the leaves (Photo: Natur-time Worldpress, Arboretum Santa Cruz, 2013).

2.2.8. Inflorescence

Typical for *Puya* genus is presence of sterile apex. Basal part of inflorescence from (1.6 – 3.2 cm) is sterile. The shape of inflorescence is usually wide and triangular, about (19.5 – 28 cm) long. The rachis is protected by dense cover of trichomes. Bracts are brown, membranous and the size is about (8.5 – 12.5 x 3.5 cm). Floral bracts can be elliptical or sometimes can be ovate, floral bracts exceed the sepals (2 - 5 x 0.7 – 1.4 cm). Size of pedicels is (5 – 12 x 1.5 – 2.5 mm). As shown in Figure 5, the giant flower spike produces clusters (Zizka *et al.,* 2013).

Flower size can be different, but still is in the range of (2.7 – 5.2 cm) long. Range of flower color is extensive from metallic – turquoise to green and blue-violet. *Puya berteroniana* is in blossom during the late spring and early summer (April, May, June, July and August). Flowers are clustered at the top (see Figure 5,C). The flower head is about 1m long and overflowed by racemes of thickset flowers, the sterile tips of which stick out (Zizka *et al.,* 2013). The bright orange stamens play important role in attracting of pollinators. Because exclude high quality nectar, which is very useful for the birdies, who can perch on sterile horizontal stem (Hornung – Leoni *et al.,* 2013).
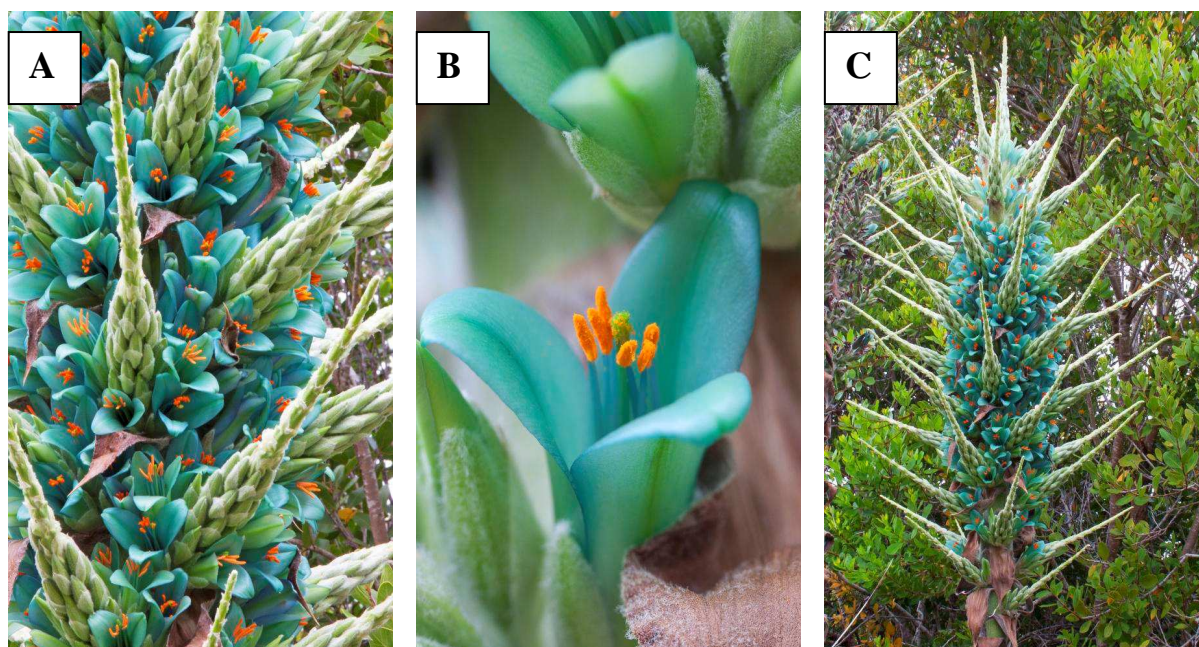
Figure 5. Blue *Puya berteroniana* flowers at the University of California– Santa Cruz Arboretum. A – group of flowers of *P. berteroniana*, B – detail of orange stamens in bloom, C – giant flower spike (photo: Natur-time Worldpress, Santa Cruz Arboretum, 2013).

Shape of sepals is rounded but sometimes we can recognize the acuminate shape of sepals. Sepal size is (1.8 – 4 x 0.4 – 0.6 cm). Petals are (4.5 cm) long and filaments are usually about (3 cm) long (Zizka *et al.,* 2013). Ovary size achieves about (6 x 3 mm). In the case of *Puya* species the style and stigma are not visible. Coloration of pollen is yellow. This pollen is transferred mainly by humming birds. In the case of this genus, the plants are usually fertile trough the whole life (Zizka *et al.,* 2013).

Puya has developed the two flowering strategies. These strategies are important for attracting of different pollinating birds. Only hovering hummingbirds can reach the nectar from tips of short branches from some species flower. The other one, bloom close to the stalk with the short branches for each flower, which is used for suck of nectar by perching birds (see Figure 6). *P. berteroniana* uses the second flowering strategy (Benzing, 2000).

Figure 6. Sucking of nectar from *P. berteroniana* by perching birds (photo:Natur-time Worldpress. Santa Cruz Arboretum, 2013).

2.2.9. Fruit, seeds and nectar

There is no study about fruit and seed up to know. Seed are usually fertile and vital and thanks to this fertility can be used mainly for vegetative propagation (Zizka *et al.,* 2013). *Puya* represent an excellent model of plant morphology and concentration of hexose rich nectar, which is very important for diversity of pollinators (Gonzáles-Goméz and Valdivia, 2005; Salinas *et al.,* 2007) thanks to high interspecific variation of nectar characteristics (Benzing, 2000). The nectar traits are different mainly in sugar type, concentration and volume between plants, that are pollinated by special nectarivores birds or plants, that are pollinated by generalists (Johnson and Nicolson, 2008; Brown *et al.,* 2009; Brown *et al.*, 2011). The volume of the nectar is higher in the subgenus *Puya* than in subgenus *Puyopsis*. There are also some differences in amount of nectar between certain species (Hornung-Leoni *et al.,* 2013). It the *P. raimondii* and *P. chilensis* exit possibilities of limiting nectar evaporation or can allow the movement of water into the plant nectar (Johnson and Nicolson, 2008), but in the case of *P.coeruela* and *P. venusta* there is a smaller volume of nectar and the trait can present evaporation too (Baker, 1978).

2.2.10. Root system

P. berteroniana has fully – functionally developed root system. Roots can uptake nutrients from soil as opposed to air and water can be obtain from deep water level (Dallman, 1998). The roots are usually thick-walled and suberized, which is very important for terrestrial *Puya* species (Wan *et al.,* 1994). There are also possibilities of creating the "rainy roots" such for agave, cacti and some desert shrubs, in which are developed as following short pulses of rain events (Benzing, 2000; Nobel, 2003; Schwinning and Salas, 2004).

2.2.11. Uses and properties

Species from family Bromeliaceae are mainly used as ornamental plant (Kunte and Zelený, 2009) because of the interesting production of high number of flowers per inflorescence (Foster, 1950).

In general, *P. berteroniana* is mainly planted as an ornamental plant due to its attractiveness of amazing aqua – blue flowers and can be planted for its interesting and stunning flower spike. *P. berteroniana* has a great ornamental value. This plant can be also cultivated as edible plant. It is also very suitable for landscape method xeriscarping, which is developed for arid and semi-arid areas, where is applied the water conserving techniques (Encyclopedia Britannica, 2014). It can be planted in rocks gardens as a bold accent or in front of tall species from family Cactaceae for their contrast between them. Main uses are architectural, can be use as container plant, in greenhouses for their tropical effect. *Puyas* are very popular in landscape of southwest desert and in California (Benzing, 2000).

From common species *P. chilensis* are used the bases of young leaves for making salads. In some part of Peru – Chumbivilcas, the inflorescences of *Puya* species are burned and its ash is usually prepared with a sugar as a meal called "Tocra" or "Illipta". This mix is commonly consumed by the local people. In other parts of Peru the centre of inflorescence is dried and used as a powder. The powder is usually used as a flavor (Leiva *et al.,* 1991). Beverage called "Chica" is prepared from pulp of inflorescence, where the pulp has to be roasted, and fermentated (Leiva *et al.,* 1991). Chica is consumed in traditional occasions. Some species as *P. raimondii* can be use in traditional ceremonies and festivals in Peru (Leiva *et al.,* 1991).

Inflorescence of some species is used as fodder for animal and also can be used as nature food for bears (Chung, 2004; Goldsteins and Salas, 1993). Young leaves are grazing by cattle and sheep (Venero, 1984). The sterile apex of *Puya* inflorescence and erect stem are specific trait of this species, this sterile apex can be also used as feeding station for perching and humming birds (Baker and Baker, 1990; Scogin and Freeman, 1984; Foster, 1950; Johow, 1898)

From dry parts of plants can be produced the plant fuel (Venero, 1984). Leaves are used in buildings – as a roof of houses also for shelter and keep the cattle.

Dry inflorescence with other components is used for constructing of walls or doors (Villinger, 1981; Venero, 1984).

When the plants release the seeds, the giant rosette is usually burnt and rachis must be dried. Tool bases can be made from thin roots of dry plants in Checayani, Peru (Leiva *et al.,* 1991). Wool hats are made from resins (Leiva *et al.,* 1991).

Fibres from the leaves of *P. chilensis* are very often utilized for making fishing nets (Mabbarley, 1975).

2.3. Plant husbandry

2.3.1. Cultivation

Cultivation of *Puya* species became very popular in last few years, especially in countries and gardens of South and Central America. A lot of *Puya* species are found in wild nature, not in cultivation. These plants are cultivated mainly for their ornamental value and purposes. *Puyas* are perennial and sometimes rare species which requires sunny and sheltered position. These plants are usually cultivated very similarly as other ornamental plants from family Bromeliaceae and also like plants from Cactaceae family (Steens, 2011).

It is relatively easy to grow these plants. There are also some possibilities to use this plant in intercropping system with some tuber crops and cereals. For example, *P. raimondii* is intercropped with cassava (*Manihot esculenta*) and different varieties of maize (*Zea mays*) in Colombia and Venezuela (Borsdorf and Stadel, 2013). In intercropping system, *Puya* has one big disadvantage – its size. It is very difficult to find the space for normal growing without some restriction. Also the spines and sharp edge of leaves can be the barrier for managing (Borsdorf and Stadel, 2013).

2.3.2. Propagation

In general, most of Bromeliads are usually easy to propagate. Majority of them is propagated by offsets like a *Tillandsia, Vriesea* or *Aechmea* species. In the case of *P. berteroniana*, there are two methods of propagation.

The first way is an asexual reproduction by offsets, where we can obtain clones. The second method is sexual reproduction, where the plants are propagated by the seeds.

Propagation by seed is more common. However, sexual way of reproduction is slower and more difficult process than asexual reproduction (Zizka *et al.,* 2013).

Propagation by pups depends on a bract, which plays very important role in asexual reproduction. When the bracts die, the healthy mother plants can produce a large amount of pups during flowering. The pups usually look like as tiny version of mother plants. The pups are removed from mother plant after their developing of small rosette of leaves similar to mother plant. After removing, the pups grow separately.

For example, the vegetative propagation of *P. mirabilis* can be very useful. Its florel can be used to provide of flowering, which can be influenced by ethylene. The use of ethylene can lead to provide higher and easier production (Pertuit, 1995).

Propagation by seeds is slower process. It is a crossing of two plants and sprout of the seeds (Steens, 2011). Seeds are spread to short distance by wind (Steens, 2011). Seeds may be collected from mature mother plants and they can be sown directly on surface of substrate – mainly in envelopes or containers, where it is necessary to maintain high volume of humidity and prevent against drying out. They are stored in cool box or in cool room, refrigerate seeds are utilize for long – term storage. These plants have no dormancy, which would be necessary to break it. Fresh seed germinates within 14 days after sowing, but time of germination can be different according to conditions (Steens, 2011).

2.3.3. Planting

Puya species are planted into big holes or beds, but it is not necessary to plant them in deep. Appropriate soils are gravel soils, sandy soils with organic matter.

Keeping of root ball intact is very important. Space around roots must be filled by lightly soil. Cover is spread over by leaf or bark mulch around 1-3 inches thick, but the mulch can not reach of height of stem (Steens, 2011).

2.3.4. Fertilization

In general, highly flowering plants requires fertilizer with high content of phosphorus. For production of leaves is usually used the fertilizer with high content of nitrogen. An appropriate fertilizer is classical NPK. For *P. berteroniana* can be used the water soluble or granulated fertilizer (Steens, 2011).

The granulated fertilizers in small amount are applied under plants from the stem to beyond the outer spread of branches or foliage. It is necessary to fertilize early in the plant´s growing cycle (Steens, 2011).

2.3.5. Pests, diseases and human factors

In general, *Puya* genus is mostly resilient to pests and diseases (Rees, 2004). There are no special types of disease, which occur in the *Puya* genus. Scale insect can be dangerous for growth of plant, especially when the scale insect sit on bottom site of the leaves and exclude the honeydew. For example, *P. raimondii* showed a little genetic variability between population, therefore these communities can became highly susceptible to pest and disease (Dearringer, 2013).

Nowadays, there are also human factors, which can influence the plants – mainly the highest bromeliad – *P. raimondii*. The largest threat for cultivation is a grazing in highlands. Heavy cattle easily trample seedling and will make growth and germinating impossible. The other big problem is also fire, which is usually used for maintaining pasture land (Dearringer, 2013).

2.4. Variability

2.4.1. Morphological variability

Puya's variability is mainly recognized by the morphological variation (Hornung-Leoni and Sosa, 2004). There are groups of plants divided according to color of petals (Zizka *et al.,* 2013).

Morphological characteristics are based on color of petals, different groups of size of branches, the size of leaves, size of flowers and according to inflorescence. These characteristics are usually allometrically related (White, 1983; Primarck, 1987; Maintre and Midgley, 1991). Allometric growth of plant is defined as different growth of various parts of plant parts (Niklas, 1994) and according to changes in the shape, which is connected with the changes in plant size (see Figure 7) (Booksteins, 1997).

In the present, we can find a lot of studies dealing with allometric relationship among parts of plants. In allometric studies are usually used the correlation between the size of plant and size of inflorescence, this correlation is explicated and attributed to pollinator visits (Donnelly *et al.,* 1998; Elle and Carnes, 2003).

Different pollinators have the contact with greater number of flowers usually in larger inflorescence than in small inflorescence (Wilson and Price, 1977). Other correlation is between number of seeds and the plant size, which also depend on the different pollinators (Robertson and Macnair, 1995). As different pollinators means the humming birds, perching birds, bees, bats and moths, which are typical for *Puya* genus (Benzig, 2000; Varadarajan and Brown, 1988). Petal size is connected with number of pollinators. For example, when increase the visits of pollinators, also the size of plant increase (Delph, 1994). Variability of size and flowers is closely connected with different pollinator or breeding system (Ornduff, 1969; Faegri and Von der Pijl, 1971). Some recent studies suppose, that also exist the correlation between size of the plants and altitude in some geographical areas (Hedberg, 1970; Mabbarley 1972; Hornung - Leoni and Sosa, 2004). As shown in Figure 7, there are a lot of morphological characteristics for allometric studies.

The morphological variation across the range of distribution is relatively huge. Plant size also increases with higher elevations for example we can mention the largest bromeliad - *P. raimondii* (Foster, 1950). This species has lack sufficient variability in genome than other *Puya* species (Foster, 1950).

In comparison of *P. boliviensis*, which is quite similar to *P. chilensis*, the plant is smaller in all plant parts and inflorescence usually bears less branches. The other example is comparison of *P. berteroniana* and *P. alpestris*, where the *P. berteroniana* has inflorescence with about 50 to 100 branches and *P. alpestris* has inflorescence with 15 to 20 – 30 branches (Hornung –Leoni and Sosa, 2005).

The size of both plants is different too. *P. berteroniana* has usually 2 – 5 m in heigh and *P. alpestris* has usually 1 – 2 (2.5) m in height. In this comparison, there is also big difference in geographical range (Hornung-Leoni and Sosa, 2005).
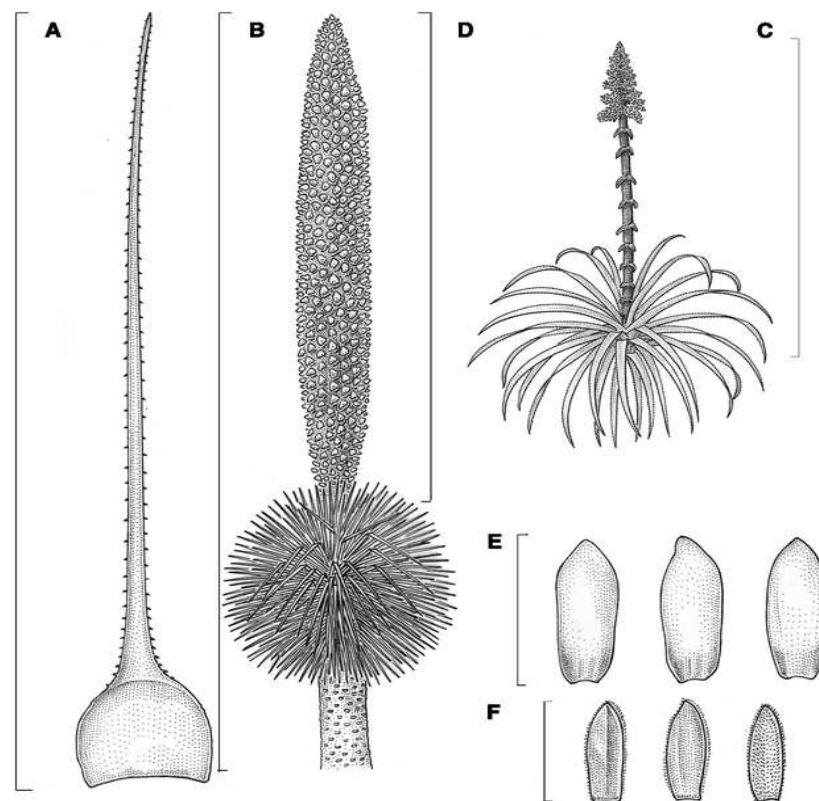
Figure 7. Characteristic of plant for allometric studies – A) length of leaves, B) height of plant with stem, C) total height of stem, D) length of inflorescence, E) length of petals, F) length of sepals (Hornung – Leoni and Sosa, 2005).

P. berteroniana is connected with more arid bioclimate types and area with sclerophyllous shrubs and *P. alpestris* is conned with humid seasonal bioclimate types and ares with sclerophyllous forest of southern Chile (Luebert and Pliscoff, 2006; Zizka *et al.,* 2009). Other very variable group is *P. coeruela* that is restricted to the Mediterranean climates and Matoral vegetation (Zizka *et al.,* 2009).

Different groups that are divided according to color of flower occur in highly variable taxon, but these groups are not taxonomically recognized up to now.

P. coeruela is mainly restricted to the coastal Cordillera, but the other are primarily found on western parts of the Andes. On of the most easily recognized species is *P. chilensis*, which has the similar height as *P. berteroniana*. *P. boliviensis* is only one species geographically isolated from other species, mainly is found in coast of Quebradas. Other species with restricted distribution is *P. gilmartiniae* (Varadarajan and Flores, 1990).

2.4.2. Phylogenetic variability

Group of *Puya* plant is classical example of rapid diversification in Andes (Schulte *et al.,* 2010; Givnish *et al.,* 2011). *Puya* genus is one from several genera from family Bromeliaceae, which is very variable in the ability of different species to produce big amount of offsets (Barbará *et al.,* 2009). Phylogenetic study according to Jabaily and Sytsma (2010) show, that *Puya* uncovered two major clades (Core *Puya* and *Puya)*, which are supported by using a combination of plastid DNA and nuclear single – copy gene sequences (Jabaily and Sytsma, 2012). The most important phylogenetic issues connected with *Puya* species are only two. The first one is an emerging phylogenetic records place the large genus of *Puya* species with dehiscent fruits and the second one is, that *Puya* shows a big species radiation, which includes morphological and ecological variation.

2.5. Use of *in vitro* technologies

Plant tissue culture research is active for many decades and leads to develop a lot of techniques *in vitro*. Possibility of plant cloning by using of *in vitro* methods is probably known from 1940 (Thorpe, 2006; White, 1963). *In vitro* propagation of ornamental plants by mass production of plant material – clonal propagules has been known since 1970 (De Fossard, 1986; Knauss, 1976). Micropropagation via plant tissue is main technique for producing of plants called the clones.

There are three main ways, how to promote these clones: micropropagation of shoots or buds; following shoot morphogenesis and somatic embryogenesis (White, 1963). Plant species diversity dramatically increased during the last centuries and nowadays we can recognize over 500 millions of plant species in which the *in vitro* techniques are used, where from 50 to 70% of these plants are ornamentals plants. Plant genetic resources are mainly conserved for their future in improvement programs for germplasm conservation (Ashmore, 1998). Nowadays, these techniquesare commercially used for propagation and improving a wide range of plants from commercially used crops to ornamentals plants. Human pressure permanently increases and climate changes provide production constraints, for this reason is very important to use plant tissue culture for developing of sustainable production system, which should be environmental stable and have to be human health perspective (Altman and Meiri, 1998). The most important task is "*in vitro* propagation" (Pence *et al.,* 2002), slow-growth storage, cryopreservation of germplasm (Taylor *et al.,* 2010), production of seeds and safety of germplasm (Pence *et al.,* 2002). These techniques are very important in plant improvement and genetic engineering (Altman and Meiri, 1998). Possibilities of genetic engineering are in limit range from pharmaceutical industry to agriculture. According to Altman and Meiri (1998) *in vitro* tissue cultures have a lot of advantage aspects, but one of the most important is mass propagation of plant material. These biotechnological methods lead to maintain and assessment of germplasm (Golmirzaie and Salazar, 1995). *In vitro* techniques are very usefull tool in maintenance of the uniformity among the progeny, preservation of health plant genotype and reduction of possible disease (Sharma *et al.,* 2007).

2.5.1. *In vitro* technologies in Bromeliaceae

Bromeliads are of the bases of the ornamental industry worldwide, being commercialized according to the features and color of the foliage and fascinating flowers, therefore is very important to keep the genetic variability and keep the population viable for next several centuries (Jain and Ochatt, 2009). The wide range of *in vitro* technologies is used in the family Bromeliaceae.

Tissue culture techniques provide the valuable tools for the mass propagation of many bromeliads that are rare or threatened with extinction (Pompelli and Guerra, 2004). Seeds of Bromeliads are used as explants, which represent the genetic structure of target population for conservation (Mercier and Kerbauy, 1997). Multiplication of bromeliads by method of micropropagation is very efficient technique that providing shoots growth from the axil (Fitchet, 1990; Hosoki and Asahira, 1980). Use of regenerating system, which is not associated with formation of callus structure is highly recommended for germplasm conservation of bromeliads, where the main goal is minimize the risk of potential genetic alternations (Villalobos *et al.,* 1991). Many bromeliads are micropropagated by seeds, for providing of genotypic variety (Soares dos Santos *et al.,* 2010). As show in Table 2, the various plant biotechnologies in Bromeliaceae applications were used under *in vitro* conditions (Tab.2).

Table 2. Plant biotechnology applications for the conservation and use of genetic resources of selected bromeliads : *Puya santossi,Guzmania, Tillandsia cyane* and *Vriesea reitzii*

Biotechnology application	*Guzmania* spp	*Puya santossi*	*Tyllandsia cyanea*	*Vriesea reitzii*
Micropropagation	Huang *et al.,* 2011	Pedroza – Marique and Tibocha, 2008	Pierik, 1991	Alves *et al.,* 2006
Organogenesis	Huang *et al.,* 2011	Pedroza – Marique and Tibocha, 2008	-	-
Callus culture	-	-	Merers, 1977	Alves *et al.,* 2006
Polyploidization	-	-	Soltis *et al.,*1987	-
Cryopreservation	-	-	Parton *et al.,* 2002	Rech Filho *et al.,* 2005
Transgenic plant	-	-	Synková *et al.,* 2006	-

In vitro conservation of bromeliads at low temperature can be inexpensive but at this point not without risk. Cryopreservation is still experimental for several years therefore a few taxa have been tested after long-term storage (Benzing 2000; Hamilton 1992). However, advances in the general procedures and methodologies are improving these techniques for the conservation of endangered plant species (Garliardi, 2003).

For Chilean bromeliads germplasm conservation are used the criterias and categories of International Union for Conserving of Nature and Natural resources (IUCN). In the case of Chilean bromeliads were mainly used these categories: critically endangered (CE), endangered (EN), vulnerable (VU), near threatened (NT) and least concern (LC) (Zizka *et al.,* 2009).

For *Puya santossi* was cultivated on full strenght medium MS (Murashige and Skoog, 1962) containing full of the salt, with addition of 100 mg.l^{-1} *myo*-inositol, 15 g.l^{-1} sucrose and 8 g.l^{-1} agar as solidifying agent and pH of MS was adjusted to 5.7 by KOH and acetic acid.

In addition to its important role in genetic resource storage, *in vitro* technologies play a major role in the international distribution of clonal genetic resources (Bortlová, 2012).

In vitro initiation of plantlets in bromeliads is a very useful tool for mass production mainly for *Aechmea fasciata* (Vinterhalter and Vinterhalter, 1994), *Dyckia dystachya* (Pompelli and Guerra, 2005), *Dyckia macedoi* (Mercier and Kerbauy, 1993), *Aechmea fulgens* (Pierik and Sprenkels, 1989*), Cryptanthus bromelioides* (Matthews and Rao, 1982), and *Tillandsia cyanea* (Pierik and Sprenkels, 1991). Propagation by direct morphogenesis has been previously reported in many ornamental plants such as *Anthurium* (Preil, 2003), *Dendrobium stronghylantum* (Kong *et al.,* 2007), *Aerides maculosum* Lindl. (Murthy and Pyati, 2000). Some other bromeliad species have been tissue cultured because of their rare or endangered status, classical example is *Puya tuberosa* (Varadarajan *et al.,* 1993) and *Tylandsia dyeriana* (Rogers, 1984).

2.5.2. Use of *in vitro* technologies in *Puya* genus

In vitro techniques in bromeliads have been tested mainly for germplasm conservation and for using in commercial production. *In vitro* applications include planting of genetic material as explants, mainly cultivated from seeds and buds in test tubes containing supporting medium for growth and development of explants (Seminario *et al.,* 2003). Unfavorable conditions and occurrence of diseases can cause the risk of losing of germplasm in the field.

Plant biotechnology offers an alternative for commercial production of bromeliads trough *in vitro* technologies (Golmirzaie and Salazar, 1995). In general, micropropagation offers opportunities for commercial production of many true-to-type of *Puya* species and others genus in the short time period. Micropropagation was used in species *P. santossi*, where the Pedroza-Manrique and Tibocha (2008) established the full strength MS medium as appropriate for cultivation. They tested eight different media with different concentrations of indole-3-butyric acid (IBA) and 6-benzylaminopurine (BAP) for inducing of shoots and choose the most appropriate one. The temperature for cultivation was established at 23 °C and the photoperiod was established for 12 hours under the fluorescent light FL – 20D/18, 20W, China Electric Co., Taipei. For rooting was used the 12 different media supplemented with NAA, 2,4 – D (2,4-dichlorophenoxyacetic acid) and kinetin (Pedroza – Manrique and Tibocha, 2008).

2.6. Molecular markers

The use of molecular markers in the last few decades play important role in biotechnology and genetic studies. There is a wide scale of different types of molecular markers. These types of molecular markers are divided into three important groups: morphological molecular markers, DNA – based molecular markers, biochemical molecular markers (Kumar *et al.,* 2009).

Molecular markers are divided into two groups: based on non – PCR (Polymerase chain reaction) includes:

RFLP (restriction fragment length polymorphism) and based on PCR includes RAPD (Random amplified polymorphic deoxyribonucleic acid, AFLP (Amplified fragment length polymorphism), ISSR (Inter simple sequence repeats), SSR (Simple sequence repeat), SNP (Single nucleotide polymorphism) etc. (Weising *et al.,* 2005).

Nowadays, in plant breeding is mostly used a marker-assisted selection (MAS) (Henry, 1998). The germplasm characterization, analysis and identification of somatic plant hybrids, purity of the seed and identity of plant material in production (Henry, 1998) are included in other applications.

On the other side we can use the molecular markers in genetic populations, in molecular taxonomy, in analysis of somatic mutations (Zietkiewicz *et al.,* 1994) and also in mapping of genome (Reddy *et al.,* 2002; Zietkiewicz *et al.,* 1994). Molecular markers can be very useful in evolution and phylogenetics and in genetic diversity research (FAO, 2005; Reddy *et al.,* 2002).

The DNA–based molecular markers are more reliable thanks to high polymorphic character and heritable patterns (FAO, 2005; Chawla, 2002). During the last few years, application of molecular markers became more cheap and simple. Application of molecular markers in plant biotechnologies of tropical and subtropical ornamental species permanently increases, for these plants are available only limited research resources (Henry, 1998).

2.6.1. Use of molecular markers in Bromeliaceae

The wide range of genetic diversity was assessed in these plants of family Bromeliaceae by using of molecular markers: *Aechmea fulgens* (Almeida *et al.,* 2012), *Ananas comosus* (Wöhrmann and Weising, 2011), *Neoregelia, Guzmania* and *Vriesea* (Zhang *et al.,* 2012), *Aechmea gomosepala* (Zhang *et al.,* 2012), *Vriesea cacuminis* (Ribeiro *et al.,* 2013*), Puya raimodii* (Sgorbati *et al.,* 2004), *Tillandsia califanii* and *T. tomasellii* (De Castro *et al.,* 2009), *Vriesea gigantea* and *Alcantarea imperialis* (Palma – Silva *et al.,* 2007).

The microsatellites were used for assessment of the role of clonal reproduction in the *Alcantarea geniculata* and *A. imperialis*. The data from this evaluation indicating,

that the hybridization and reticulation involves the high – altitude species in the parts of co – occurrence (Barbará *et al.,* 2009).

Zhang *et al.* (2012) investigated the genetic diversity in hybrids of *Aechmea gomosepala* and *Aechmea recurvata*. For this purposes were used the inflorescence characteristics and SRAP. According this analysis, the putative hybrids of these plants were intermediate between both of parental plant material.

According to Ribeirro *et al.* (2013) the *Vriesea cacuminis* is endemic species, native to Brazil. *V. cacuminis* is designated as a "vulnerable" species according to IUCN. They evaluated the genetic diversity of 70 individual species of *V. cacuminis* by using of 16 ISSR molecular markers.

Puya raimodii was analyzed by (Sgorbati *et al.,* 2004) by using of molecular markers. This investigation team evaluated 14 genotypes out of 160 plants of this species.

A phylogenetic relationship between two *Tillandsia* species – *T. califanni* and *T. tomasellii* was evaluated by the nucleotide sequence from 6 regions of chloroplast genome (De Castro *et al.,* 2009).

In the case of *Vriesea gigantea* and *Alcantarea imperialis* were used 15 polymorphic microsatellites markers. For each locus was evaluated number of alleles from 3 to 16 (Palma- Silva *et al.,* 2007).

Ananas comosus was analyzed for simple sequence repeats. According to PCR was expected, that the size range will be high and they obtained 36 primer pairs (Wöhrmann and Weising, 2011; Feng *et al.,* 2013).

The primers used to amplification of microsatellites loci. Size range was from 110 to 450 base pairs (bp). The transferability of molecular markers was estimated in *Guzmania*, *Pitcairnia* and *Aechmea* species (Almeida *et al.,* 2012).

2.6.2. Use of molecular markers in *Puya* genus

Genetic diversity of *P. berteroniana* has not been described yet by using molecular marker methods. Classical example and case study is from this genus is *P. raimondii,* where were used the molecular markers for assessment of genetic diversity. In this evaluation was used the AFLP, where was used four selective primer combinations (Hornung-Leoni *et al.,* 2013).

It was evaluated for 60 individuals from different part of Peru. This case study from Peru show, that the genetic variability in *P. raimondii* is relatively high (0.935 and 0.977) (Hornung-Leoni *et al.,* 2013).

2.7. Evaluation of genetic stability *in vitro* regenerants by molecular markers

Genetic stability is a term, which is used for measuring of individual genotypes in population. Evaluation of genetic stability plays the most important role in detecting of no differences among the tested regenerated plants under the sterile conditions (Maritano *et al.,* 2010). The establishment of genetic fidelity of *in vitro* regenerated plants will be essential requisite for large scale multiplication of plants (Das *et al.,* 2010). The advantages of *in vitro* propagation is in observing of genetic fidelity in regenerants, where is necessary to establish a functional system that produce genetically stable and identical plant to the mother plant (Das *et al.,* 2010). The direct morphogenesis is based on propagation, treating and storing of plant undet the specific conditions to keep the genetic stability. Nowadays, we can find a lot of different types of clonal propagation, where the using of plant tissue culture with perfomed meristem significantly increases the genetic stability of *in vitro* regenerated plants (Mallón *et al.,* 2010). Several methods can be used to evaluate the genetic stability of *in vitro* derived clones, but most have limitations. Karyological analysis, for example, can not detect the alterations in specific genes or small chromosomal rearrangements in genome (Isabel *et al.,* 1993)

Using of different plant growth regulators such as cytokinins in the growing medium can promote the probability of mutations and variability in regenerated plants (Bairu *et al.,* 2006; Mallón *et al.,* 2010), which is known as process called the somaclonal variation (Larkin and Scowcroft, 1981).

Possible occurrence of this somaclonal variation makes the assessment of genetic fidelity very important. The occurrence of genetic defects in plants arising via somaclonal variation can limit the utility of micropropagation systems (Salvi *et al.,* 2002). As a somaclonal variation we can consider the different changes in plant karyotype, in structure of chromosome, in gene mutations and nuclear changes (Chawla, 2002).

In the plants, where the somaclonal variation is not common, these plants are free from in – meristem tip cultures.

These types of meristem can stay stable during the *in vitro* cultivation (Chawla, 2002; Reed *et al.,* 2004). In the plants, where the somaclonal variation is not common, these plants are free from in – meristem tip cultures. These types of meristem can stay stable during the *in vitro* cultivation (Chawla, 2002; Reed *et al.,* 2004).

In the plants, where the somaclonal variation is not common, these plants are free from in – meristem tip cultures. These types of meristem can stay stable during the *in vitro* cultivation (Chawla, 2002; Reed *et al.,* 2004). Nowadays, for plant breeding programmes is typical and desirable utilization of somaclonal variation. Somaclonal variation can be used in many different breeding goals (Mrhalová, 2013). This variation can brings a lot of aadvantages as: improving of plant development, improving of disease resistance of plants but on the other side can offer source of different insect, herbicides and abiotic stress (Chawla, 2002). The most important is genom rearrangement during whole cultivation, which can allow to providing new chances for alien gene introgression (Chawla, 2002; Karp, 1994). For this purposes are highly recommended using of molecular marker methods that provide information about uniformity of genome (Sharma *et al.,* 2007).

The most reliable way how to evaluate the genetic stability is use of molecular markers. Molecular markers provide quick and reasonable way. Molecular markers provide very effective tools and many applications (Sharma *et al.,* 2007).

Most of studies about genetic stability include the evaluation by using of molecular markers as AFLP, ISSR and RAPD, or combination. ISSR markers are often more reliable than fro example RAPD markers, because the former methods require more stringent PCR conditions. The ISSR markers have the locus specificity, whre the small-sized bands exhibited in a single amplification (Weising *et al.,* 2005).

Nowadays, there are a many studies in which molecular markers have been tested on *in vitro* propagated bromeliad plants (Sgorbati *et al*., 2004; Almeida *et al.,* 2012; Wöhrmann and Weising, 2011). In many of these studies the genetic stability was not detected, but occurrence of polymorphic bands was usuall. According to Sgorbati *et al.* (2004)

In other imporatant species from Bromeliaceae family, such as *Guzmania, Pitcairnia* and *Aechmea* species the assessment of polymorphic loci for SSR and ISSR molecular markers were from 80% to 90.3% (Almeida *et al.,* 2012). As shown in Table 3, various molecular markers techniques were used for determining of genetic stability in plants.

Table 3. Various plant species with presence of genetic stability assessed by various molecular markers

Species	Family	Process	Source of explants	Detection method	Stability	References
Ananas comosus	Bromeliaceae	shoot regeneration	leaves	**RAPD**	no	Santos *et al*, 2008.
Asparagus officinalis	Asparagaceae	callus culture	lateral buds	**AFLP**	yes	Pontaroli and Camadro, 2005
Bambusa nutans	Poaceae	axillary shoot proliferation	nodal segments	**ISSR**	yes	Negi and Saxena, 2011
Cannabis sativa	Cannabiaceae	Shoot regeneration	axillary buds	**ISSR**	yes	Lata *et al*, 2010.
Musa spp	Musaceae	Shoot organogenesis	pseudostem	**RAPD**	yes	Bairu *et al*., 2006.
Platanus acerifolia	Platanaceae	Shoot regeneration	axillary buds	**ISSR**	yes	Huang *et al*, 2009
Simondsia chinensis	Simmondsiaceae	Shoot regeneration	axillary buds	**ISSR**	yes	Kumar *et al*, 2011.
Theobroma cacao	Malvaceae	embryogenesis	flower buds	**CAPS**	yes	Lopéz *et al*, 2010

3. Objectives of this thesis

The main objectives of this thesis were development of an appropriate protocol for the effective *in vitro* micropropagation of *Puya berteroniana* and detection of genetic stability of *in vitro* regenerants using of molecular markers ISSR.

The partial objectives followed the certain specific goals:

- Establishment of plant material (*P. berteroniana*) into the conditions *in vitro*.

- Development of appropriate protocol for induction of direct morphogenesis in *P. berteroniana* by finding optimal concentrations and combination of plant growth regulators in cultivation medium.

- Evaluation of genetic stability of regenerated plants of *P. berteroniana* using inter simple sequence repeat (ISSR) markers and flow cytometry.

For effective micropropagation is absolutely dispensable to maintain the genetic stability of multiplied plant material. *In vitro* propagation of plant material via direct morphogenesis minimalizes the probability of occurrence of somaclonal variability. However, for this purposes the authentication of genetic stability is necessary, mainly for determination of an appropriate method of propagation.

4. Material and methods

4.1. Plant material

Seeds of *P. berteroniana* were used as an initial plant material.

4.2. Methods

4.2.1. Establishment of *in vitro* culture

The establishment of *in vitro* culture of *P. berteroniana* was carried out in the Laboratory of plant tissue culture of the Department of Crop Science and Agroforestry in the Faculty of Tropical Agriculture of the Czech University of Life Sciences in 2013 – 2014.

For sterilization was used following protocol. Seeds were surface sterilized using 70% ethanol for 2 minutes. For disinfection of seeds was tested an aqueous solution of 1% NaClO applied for 10 and 20 min, respectively. Thereafter, the seeds were three times rinsed with sterile distilled water and sown on the ½MS medium (Murashige and Skoog, 1962) with addition of 100 mg.l^{-1} *myo*-inositol, 30 g.l^{-1} sucrose and 8 g.l^{-1} agar (pH was adjusted to 5.7). Cultures were maintained at 25/23°C under a 16/8 h light/dark regime with 36 µmol m^{-2}.s^{-1} cool white fluorescent light.

4.2.2. *In vitro* propagation of plant material for experiment establishment

Firstly, sufficient plant material was necessary to ensure for the establishment of the experiment. In order to standardize the experiment, the plants for further experiment were established only from one genotype (from one seed) of *P. berteroniana*. Since *in vitro* plants did not produce shoots on medium without PGRs, according to the primary study focused on media testing, MS medium supplemented with 0.1 mg.l^{-1} BAP has been used for multiplication of plant material. Subcultivation was carried out every month and plants were cultivated under conditions decribed above.

4.2.3. Testing of media for *in vitro* propagation

The plant rosettes were cultivated on ½MS medium before transffering into the multiplication media with PGR. For multiplication experiment, plants about 1-2 cm high were used. The rosettes were cultivated on ½MS medium supplemented with 100 mg.l^{-1} *myo*-inositol, 30 g.l^{-1} sucrose, 8 g.l^{-1} agar and different PGRs at various concentrations and combinations. BAP and ZEA at concentrations 0.01 mg.l^{-1} to 0.5 mg.l^{-1} alone or in combination with NAA at concentration 0.1 mg.l^{-1} were used for the experiment. Totally, sixteen different treatments have been tested (Tab. 4). As control, ½MS medium without plant growth regulators was used.

Table 4. Treatments for *in vitro* propagation

Treatment	BAP (mg.l^{-1})	NAA (mg.l$^{-1)}$)	ZEA (mg.l^{-1})
1.	0.01		
2.	0.1		
3.	0.3		
4.	0.5		
5.	0.01	0.1	
6.	0.1	0.1	
7.	0.3	0.1	
8.	0.5	0.1	
9.			0.01
10.			0.1
11.			0.3
12.			0.5
13.		0.1	0.01
14.		0.1	0.1
15.		0.1	0.3
16.		0.1	0.5
Control plant	**0**	**0**	**0**

Cultures were maintained for 28 days at 25/23°C under a 16/8 h light/dark regime with 36 µmol m^{-2}.s^{-1} cool white fluorescent light. After 4 weeks, number of offshoots was evaluated by the statistical analysis.

All *in vitro* experiments were arranged as a completely randomized design and repeated twice (10 repetitions were in each treatment). Statistical analysis of data was performed by analysis of variance (ANOVA) and the significantly different means were identified by using the Tukey´s HSD test (p = 0.05) [StatSoft STATISTICA 12.0].

According to statistical analysis, the optimal treatment has been selected and chosen plants from this treatment were used for detection of the genetic stability using ISSR markers and flow cytometry.

4.2.4. Rooting and *ex vitro* transfer

For induction of roots were tested ten different media based on ½MS with addition of different concencentration of auxines NAA and IAA (Tab. 5). Statistical analysis of data showing *in vitro* rooting was performed by analysis of variance (ANOVA) and the significantly different means were identified by using the Tukey´s HSD test (p = 0.05) [StatSoft STATISTICA 12.0].

Table 5. Treatment used for *in vitro* rooting

Treatment	NAA (mg.l^{-1})	IAA (mg.l^{-1})
1.		0.1
2.	0.1	0.1
3.	0.1	
4.	0.2	
5.	0.3	
6.	0.5	
7.		0.2
8.		0.3
9.		0.5
Control	0	0

After 28 days, number of roots have been evaluted. After succesful rooting was necessary to carry out *ex vitro* transfer. For *ex vitro* transfer, only well rooted plants,

at least 3 cm high were used. As the substrate for the transfer of the explants was chosen classicalgarden substrate mixed with perlite in ratio 1:1.

Garden substrate was mixed with perlite and put into the glass flask covered by aluminium foil. The substrate was sterilized at 100 °C for 1 hour. The *in vitro* developed plantlets were removed from the culture vessels and transplanted into the sterilized substrate mixed with perlite and thoroughly covered with PE plastic films to maintain high humidity. After couple of days, the plants were gradually uncovered. The transplantation of explants took place in Botenical Garden of FTZ, and it was evaluated as survival percentage after one month after *ex vitro* transfer.

4.2.5. DNA extraction

DNA extraction was carried out in the Laboratory of molecular biology of the Department of Crop Sciences and Agroforestry in Tropics and Subtropics of the Faculty of Tropical AgriScience of CULS Prague in 2014. For extraction of DNA were randomly chosen ten samples from medium supplemented by 0.1 mg l^{-1} BAP and one sample without PGRs as a control plant.

For the extraction of DNA were used several types of extraction. Fisrtly, the CTAB procedure was used (Doyle and Doyle, 1987). From the fresh leaves were taken the samples about 120 mg weight. The extraction was done according to protocol of extraction: the samples were homogenized under liquid nitrogen, into the homogenized material was added the 500 µl (2x CTAB + 1% mercapthoethanol) briefly vortex and incubated at 65 °C for 45 min and centrifugated for 10 minutes on maximum. The solution was transferred into the new 1.5 ml reaction tube and for this supernatant was added by 500 µl of chloroform – IAA and vortex for 10 minutes. The water phase was transferred into the new tubes and added by the 1/5 5% CTAB and was mixed, followed by addition of 500 µl chloroform – IAA and centrifugated for 5 minutes on maximum. Water phase was transferred into the new eppendorf tubes and was added by 2/3 of isopropanol. These tubes were kept during the night in freezer at -20 °C.

After freezing the tubes were centrifugated for 5 minutes at maximum. We removed the supernatant and dried the pellet, followed by addition of 300 µl 1x TE

and keep in 37 °C, followed by addition of 20 µl 3M sodium acetate and 600 µl 96% cold ethanol, mixed 2-3 times.

For 20 minutes were kept in freezer, followed by centrifugation for 10 minutes at 4°C, the supernatant was removed and pellet was dried. Into the tubes with dried pellet was added 500 µl 70–80% cold ethanol and centrifugated at 4 °C for 10 minutes. The supernatant was removed and the cleaning step was repeated with 500 µl 70–80% cold ethanol followed by centrifugation at 4°C for 10 minutes. The supernatant was removed and pellet was dried and was dissolve in 50 µl double distilled water.

As the second method of genomic DNA extraction Invisorb® Spin Plant Mini Kit (Invitek Company, Germany) was used. For extraction were taken fresh samples about 100 mg weight. The extraction was done according to the protocol instructions; the samples were homogenized by a pestle under liquid nitrogen and transfered into a 1.5 ml reaction tube with 400 µl Lysis Buffer P and 20 µl Proteinase K, followed by briefly vortexing and incubating at 65 °C for 30 min. The lysis solution was transfered onto the Spin Filter and reaction tubes were centrifuged for 1 min at 12,000 x g. After removal of the Spin Filter, the Binding Buffer was added in amount of 200 µl, vortexed and suspension was removed onto a new Spin Filter in a 2.0 ml Receiver Tube, followed by incubation for 1 min and centrifuged at 12,000 x g for 1 min. After the centrifugation the filtrate was discarded. The phase of cleaning includes use of 550 µl Wash Buffer I or Wash Buffer II. After the addition of 550µl Wash Buffer, the samples were centrifuged at 12,000 x g for 1 min and the filtrate part was discarded. The Spin Filters with the samples were placed into a 1.5 ml Receiver Tubes. The prewarmed Elution Buffer D was added at volume of 100 µl, followed by the incubation for 3 min and centrifugation for 1 min at 10000 x g.

For third method of extraction was used CTAB protocol according to Doyle and Doyle (1987) with the modification according to (Saghai – Maroff *et al.,* 1984). The modification consisted from using of phenol used for puryfing to precipitate remaining the polysaccharides).

DNA quality was determined by 2.0% agarose gel electrophoresis and using a Nanodrop Spectrophotometer (Thermo Scientific, USA). The final concentration of all DNA samples was adjusted to 20 ηg/µl for PCR, and stored at -20 °C.

Gel was run at 55V for about 1 hour. It was visualized on a gel with a UV transilluminator and gel pictures were displayed using CSL-MICRODOC System (CLEAVER, Great Britain).

4.2.6. ISSR Analysis

A set of 6 ISSR primers (University of British Columbia, UBC, USA) were used for screening. DNA amplifications using Polymerase Chain Reaction (PCR) were carried out in a reaction volume of 20 μl with following composition: 10 μl of 2x PPP Master Mix (150 mM Tris–HCl, pH 8.8 (25 °C), 40 mM $(NH4)_2SO_4$, 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), 7.3 μl of PCR H_2O (Top-Bio, Czech Republic), 2 μl of template DNA, 0.5 μl of primer and 0.2 μl of BSA (Fermentas, Germany). The ISSR analysis was carried out by using of QB96 Server Gradient Thermal Cycler (Quanta Biotech, United Kingdom). Annealing temperatures in PCR were optimized for each primer (Tab.6).

Table 6. Conditions of PCR cycles for selected primers

Number of cycles	Step		Temperature (°C)	Duration of cycle (min)
1	Initial denaturation		94	5
40	Denaturation		94	1
	Annealing		Specific	1
	Primer	'UBC 812'	47	
		'UBC 813'	49.3	
		'UBC 814'	47	
		'UBC 824'	47	
		'UBC 834'	47	
		'UBC 841'	47	
	Elongation		72	2
1	Extension		72	2
1	Final extension		72	10

After DNA amplification, amplified products were electrophoretically separated on agarose gel, prepared from 2.0 g of agarose dissolved in 100 ml of 1% TBE buffer.

Staining capability of EtBr (Invitrogen, USA) was verified. To dye agarose gel 1 μl of each stain was used. Gels were run at 55V for about 2.5 h at 4 V.cm^{-1}. Gels were stained with SYBR® Safe DNA Gel Stain (Stratec Molecular, Germany) and the amplified stained products were visualized on a gel wit a UV transilluminator.

The gel pictures were displayed using CSLMICRODOC System (CLEAVER, Great Britain). ISSR fragments were established as a presence (1) or absence (0) of bands in the gel profile.

4.2.7. Flow Cytometry

DNA ploidy levels were detected using flow cytometry. The two-step methodology according to Doležel *et al.* (2007) was used. *Glycine max* cv. Polanka, 2C = 2.50 pg (Doležel *et al.* 1994) was used as internal standard. Approximatelly 1 cm^2 from each sample of *P. berteroniana* 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$_2$HPO$_4$.12H$_2$O) supplemented by AT-selective fluorescent dye DAPI (4´,6-diamidino-2-phenylindole) and 2-mercaptoethanol in final concentrations of 4 μg.ml^{-1} and 2 μl.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 analyzed using the FlowMax software (Partec, GmbH, Münster, Germany). Data were analyzed 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. Establishment of *in vitro* cultures and multiplication of plant material

For sterilization of plant material (seeds), the best result was achived using 70% ethanol for two minutes, followed by placing seeds into solution of 1% NaClO for 10 minutes. This process was the most effective to control contaminations in *Puya* seed culture. Using this treatment, the contamination rate varied from 0-2% seeds. The contamination was caused mainly by fungi. After successful sterilization 90% of plant material germinated. As shown in Figure 8, the seeds germinated during 8-9 weeks. In seeds, which were sterilized by 70% ethanol for two minutes and in 1% NaClO for 20 minutes, germination within 8 weeks of culture was not achived.
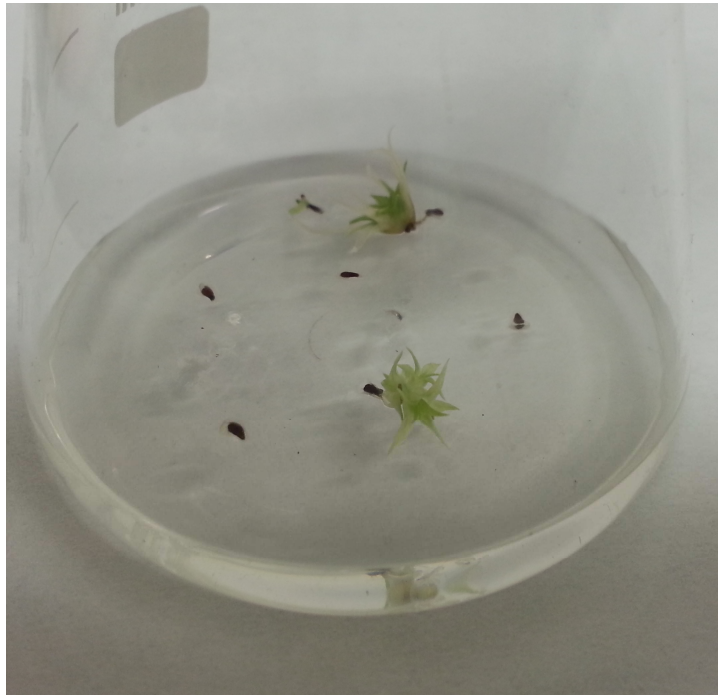

Figure 8. Germination of *P. berteroniana* within 8-9 weeks after sowing. Source: author

5.2. Direct morphogenesis

5.2.1. Evaluation of plant propagation experiment

Rosettes were transferred to different medium supplemented with PGRs (see Table 7). Sixteen different media with various concentrations of BAP and ZEA were used for *in vitro* propagation. After four weeks, explants were evaluated for an average mean shoot number per explant (see Table 7). As shown in Figure 9, A and Table 7, the most appropriate medium for proliferation and quality of *P. berteroniana* explants was the treatment ½MS supplemented only by cytokinin BAP at concentration 0.1 mg.l^{-1}. The next highest proliferation intensity was achieved in treatment ½MS medium supplemented with BAP in concentration 0.3 mg.l^{-1}. In these treatments the average means shoot number per plant was 5.46±0.90 and 4.03±0.35, respectively (see Table 7). Between these two media, no significant differences were detected. However plants on medium supplemented by 0.1 mg.l^{-1} BAP were a little bit higher and darker green than plants on medium supplemented by 0.3 mg.l^{-1} BAP. In general, lower concentrations of cytokinins provided much better results than higher concentrations. As shown in Figure 9, B, on on the full-strength MS and ½MS proliferation of rosettes was not observed.

The combination of cytokines and auxines created the secondary effect in formation of very well developed *in vitro* root. Some plant cultivated on medium with addition of NAA showed formation of roots with hairs. On the growing medium 0.5 mg.l^{-1} BAP with 0.1 mg.l^{-1} NAA and 0.01 mg.l^{-1} BAP the proliferation rate was 0%, but the quality of the explants was still very good. Nevertheless, addition of NAA in medium decreased the shoot proliferation when compared to media only with cytokinins (see Table 7). Thus, they were not suitable for *in vitro* propagation of plants.

In treatment supplemented by 0.01 mg.l^{-1} zeatin, *P. berteroniana* few explants had necrosis. The othertreatments were without the necrosis.

Overall, response of plants to zeatin was less intensive than in case of BAP. However, the plants grew up well and they were vigorous, without necrosis or vitrification.
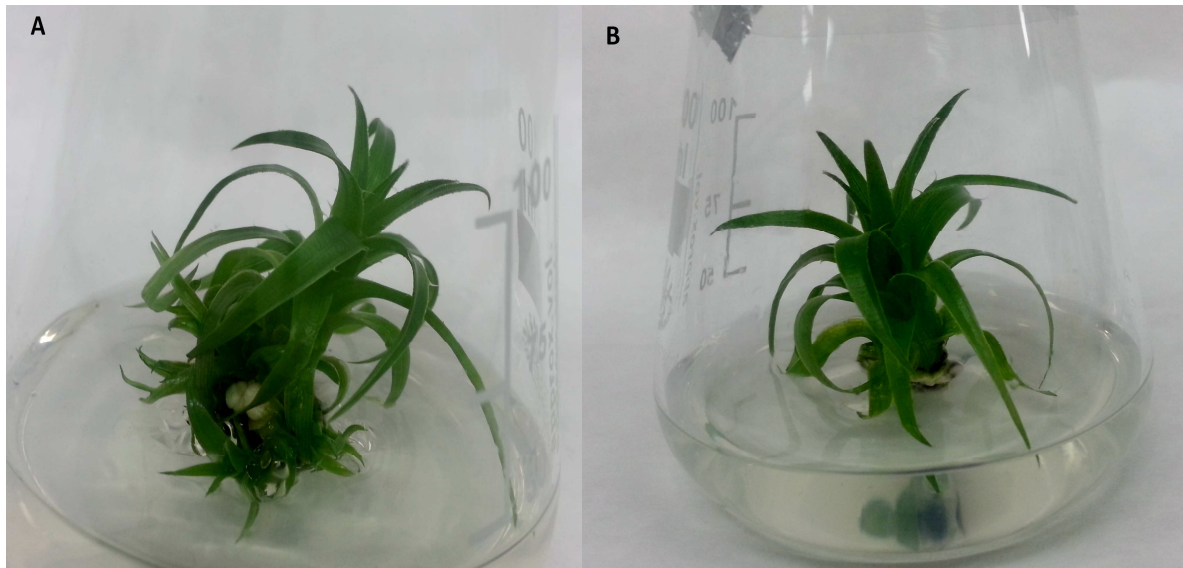
Figure 9. Explants cultivated on multiplication medium and PGRs free control medium. A – plantlets on ½MS supplemented by 0.1 mg. l^{-1} BAP.B – control plant on PGRs free ½ MS medium. Source: author

Table 7. Effects of cytokinins and auxines on multiple shoot formation of *Puya berteroniana*

Treatment	BAP (mg.l^{-1})	NAA mg.l^{-1})	ZEA (mg.l^{-1})	Mean shoot number (± S.D.) *
1.	0.01			0.00 ± 0.00
2.	0.1			5.46 ± 0.91 a
3.	0.3			4.03 ± 0.35 ab
4.	0.5			2.88 ± 0.49 bc
5.	0.01	0.1		1.40 ± 0.30 cde
6.	0.1	0.1		2.64 ± 0.47 bcd
7.	0.3	0.1		2.99 ± 0.55 bc
8.	0.5	0.1		0.00 ± 0.00
9.			0.01	0.19 ± 0.09 d
10.			0.1	2.40 ± 0.33 bcde
11.			0.3	2.40 ± 0.48 bcde
12.			0.5	2.64 ± 0.77 bcd
13.		0.1	0.01	0.96 ± 0.18 abc
14.		0.1	0.1	0.54 ± 0.17 de
15.		0.1	0.3	2.04 ± 0.58 bcde
16.		0.1	0.5	0.80 ± 0.15 cde
Control plant	0	0	0	1.19 ± 0.30 cde

* Number of shoots recorded after 28 days of cultivation

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

5.2.2. Rooting *in vitro* and *ex vitro* transfer

During January 2014, the explants cultivated on multiplication medium with the highest shoot proliferation capacity, i.e. with 0.1 mg.l^{-1} BAP, were transferred onto media supporting *in vitro* rooting (Tab. 4). After four weeks of cultivation an average number of roots were evaluated (Tab. 8).

Medium supplemented by IAA did not promote the *in vitro* rooting. Neither concentration 0.1 and 0.2 mg.l^{-1} of NAA did influence the rooting of explants. The most appropriate concentration of auxines for rooting was 0.3 mg.l^{-1} NAA, indicating that higher concentrations of NAA are more effective for root production. The roots obtained on this rooting medium rather shorter and light.

The roots of control plants were the shortest from all treatments and without branching, too (data not shown). In all treatments, there was no occurrence of branching of roots. Total appereance of explant after transfer was good without visible changes on plantlets or some necroses.

Table 8. Effects of various treatments on formation of roots in *Puya berteroniana*.

Treatment	Concentration of NAA (mg.l^{-1})	Concentration of IAA (mg.l^{-1})	Concentration of ZEA (mg.l^{-1})	Number of roots (± S.D.) *
1.		0.1		0.84 ± 0.23 c
2.	0.1	0.1		0.46 ± 0.14 c
3.	0.1			1.13 ± 0.26 c
4.	0.2			1.12 ± 0.22 c
5.	0.3			10.0 ± 0.95 a
6.	0.5			6.7 ± 0.80 b
7.		0.2		0.95 ± 0.33 c
8.		0.3		0.47 ± 0.20 c
9.		0.5		0.75 ± 0.24 c
Control plant	0	0	0	5.20 ± 0.64 b

* **Number of roots recorded after 28 days of cultivation**

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

After successful development of roots on medium containing 0.3 mg.l^{-1} NAA, the explants were transferred to *ex vitro* conditions, especially into garden substrate mixed with perlite in ratio 1:1. Only 1 explant from 30 died four weeks after transfer. The death was probably caused by fungi from badly washed agar medium.

The other plants grow normally without special requirements. A shown in Figure 10, six weeks after transfer, the 29 plants normally grow without any necroses or vitrification, providing survival rate 97%.

Figure 10. Plantlets after *ex vitro* transfer into garden substrate mixed with perlite (1:1). Source: author

5.2.3. Flow cytometry

To evaluate the effect of regeneration via direct morphogenesis on ploidy stability, we chose 10 randomly selected plants from the best propagation treatment A and subjected them to flow cytometry.

Linear histograms of relative nuclear DNA content evinced in all cases 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 (*Glycine max*) in the G0/G1 phase (see Figure 11 A; B). The DNA-ratios of *P. berteroniana in vitro* plantlets varied from 0.347 to 0.353, and they were not significantly different to that of the control plant (0.349). Flow cytometric analysis suggested that the plants after *in vitro* propagation had maintained stable ploidy level.

Absence of any alterations in ploidy level indicates that in plants obtained by direct morphogenesis not occur of somaclonal variation at the genomic level.
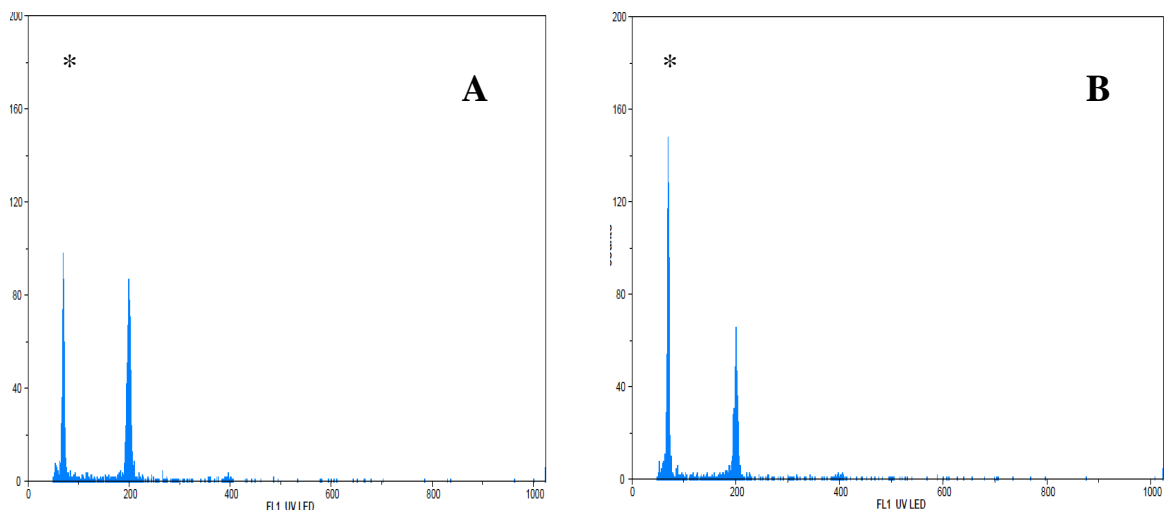

Figure 11. Representative flow cytometric histograms documenting the realite DNA content of *in vitro* plants of *P. berteroniana.* A – control plant; B- randomly selected *in vitro* plantlets. The peak indicated as "*" correspond to the internal reference standart (*Glycine max).*

5.2.4. DNA extraction

In our study we observed the problem with extraction of DNA from fresh plant samples of *P. berteroniana*.

By using classical CTAB method it was not obtained the satisfactory concentration of DNA in samples for further experiment. From ten samples, which had been extracted, the concentration reached from 0.3 to max 3 $ng.ml^{-1}$. Repetition of extraction did not provide satisfactory results.

The second method of extraction was the appliccation of the commercially available kit Invisorb Spin Plant Mini Kit (INVITEK Company, Germany). This kit showed slightly better results in concentration of DNA, which rised up to 19.9 $ng.ml^{-1}$. The other samples showed the concentration of DNA only from 3 to 9 $ng.ml^{-1}$. This method can not be considered as sufficiently effective.

According to our previous experiences, there were used the modification for CTAB protocol by applications of phenol, which was used as an additional agent for purifing to precipitate remaining the polysaccharides. By this method, we obtained the higher concentrations of DNA per ten samples than in previous two methods. The highest concentration achieved was 72 $ng.ml^{-1}$, on the other side the lowest concentration was 8.9 $ng.ml^{-1}$.These concentrations were used for assessment of annealing temperature for each primer.

The isolated DNA using CTAB and fenol was electrophoretically separated on 2% agarose gel to test the capability of DNA for using in further experiments (Fig.12).
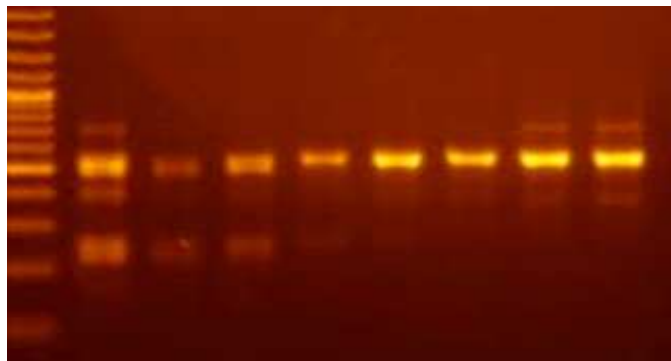

Figure 12. DNA samples screened for optimalization on 2% agarose gel to verify the capability of DNA for further experiment**.**

5.2.5 ISSR

During this preliminary study a set of 6 ISSR primers were screened. Each primer produced average number of 19 clear and reproductible amplicons. The amplified products of all 6 ISSR primers were monomorphic across the regenerated explants and were identical to the mother plant (control plant). Detailed results are summarized below (see Table 9).

Primer 'UBC 812' produced 3 scorable monomorphic bands in range 550 – 750 bp. Primer 'UBC 813' produced 3 scorable monomorphic bands per primer in range 800 – 1400 bp. Primer 'UBC 814' produced 4 scorable monomorphic bands in range 450 – 700 bp. Per primer 'UBC 824' I detect 4 scorable monomorphic bands in range 550 – 850 bp. Primer 'UBC 834' showed 3 scorable monomorphic bands in range 550 – 750 bp.

The last primer 'UBC 841' detected only 1 scorable monomorphic band on 550 bp. None of tested primers produced polymorphic bands. The example of monomorphic patterns obtained by ISSR is expressed below (see Figure 13; 14).

Table 9. ISSR primers used for detecting the genetic stability in regenerants of *P. berteroniana* and the amplicons generated (bp= base pairs).

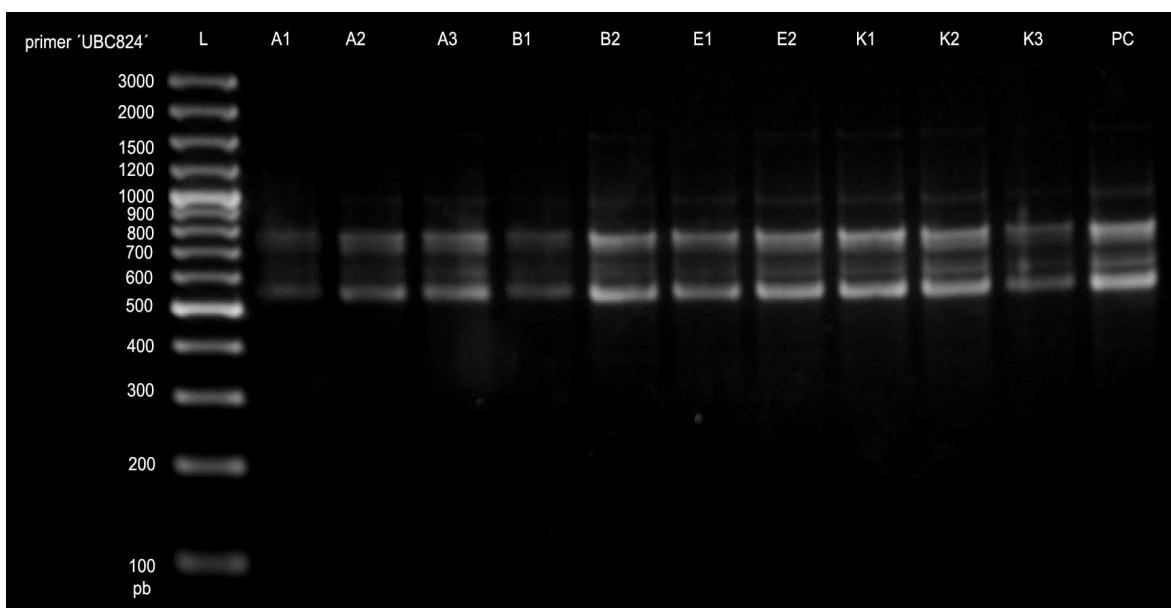| No.	Primers code	Annealing temperature (°C)	Total number of bands amplified	Number of scorable bands per primer	No. and frequency of polymorphic bands per primer	Range of amplification (bp)
1.	'UBC812'	47.0	33	3	0	550 - 750
2.	'UBC813'	49.3	33	3	0	880 - 1380
3.	'UBC814'	53.6	44	4	0	450 - 700
4.	'UBC824'	47.0	55	4	0	550 - 850
5.	'UBC834'	47.0	66	3	0	550 - 750
6.	'UBC841'	50.0	11	1	0	550
Total			**253**	**19**	**0**	

Figure 13: Monomorphic profile using primer UBC 824. bp: base pairs; L: 100 bp DNA ladder; Cp: control plant; A1-K3: regenerants from treatment with BAP in concentration 0.1 mg.l^{-1}.
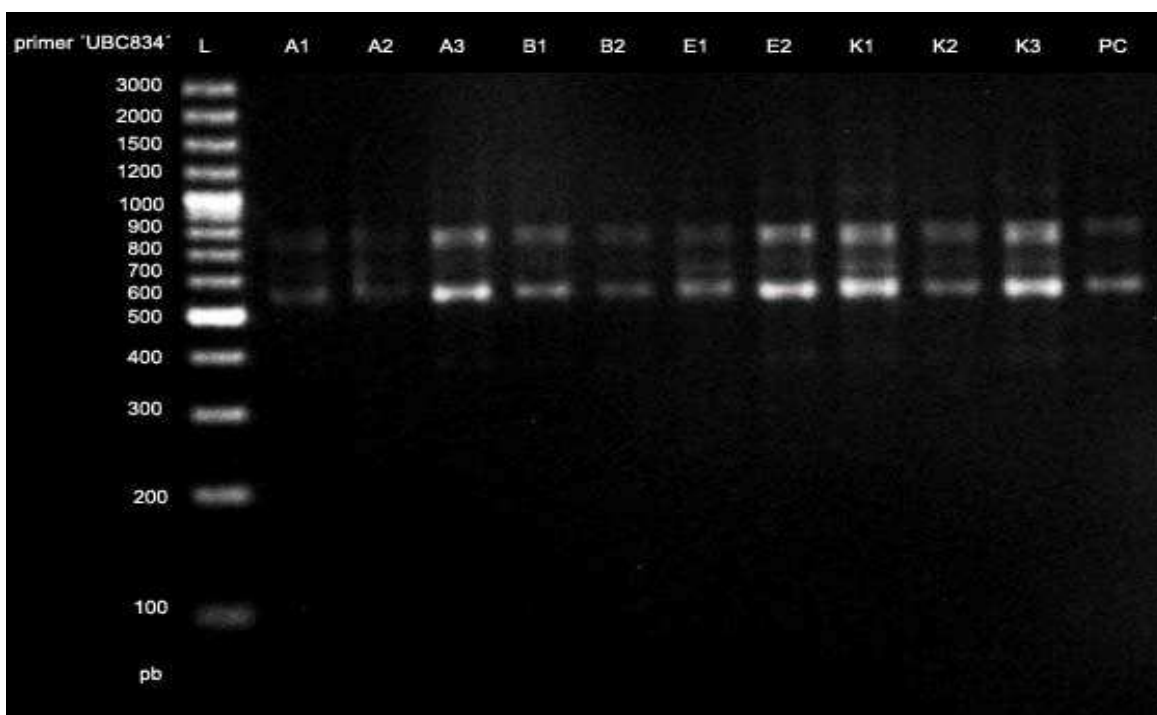

Figure 14: Monomorphic profile using primer UBC 834. bp: base pairs; L: 100 bp DNA ladder; Cp: control plant; A1-K3: regenerants regenerants from treatment with BAP in concentration 0.1 mg.l^{-1}.

6. Discussion

6.1. Sterilization of plant material

During the surface sterilization of *P. berteroniana* seeds, the use of NaClO proved to be very effective agent in contamination control. Our results on seed sterilization of *P. berteroniana* highly agreed with those of Galvanesi *et al.* (2007) for *Aechmea blanchetiana*. The fungal contamination, which was observed in many bromeliad species are very likely related to seed handling and sterilization during inoculation. Fortes *et al.* (2004) present that, despite the losses caused by fungi *in vitro*, much important is the occurrence of bacteria contamination. Preventing the fungi and bacterial occurrence highly depends on adjusting sterilization methods.

In this study, amount of contamination in *P. berteroniana* was minimal and the plantlets did not show the damage of tissue. Bhojwani (1980) and Neuner and Beiderbeck (1993) founded NaClO is the most appropriate agent for surface sterilization of *Salix caprea* (Salicaceae). Peréz *et al.* (2013) in *Hohenbergia penduliflora* (Bromeliaceae) used for sterilization 20 min in 2% NaClO. However for our plant species was proved to be better was achived using the protocol of disinfection according to study Pedroza – Marique and Tibocha (2008). The most efficient treatment in sterilization of *P. santossi* was 1% NaClO applied for 10 minutes. This treatment was the most appropriate also for *P. berteroniana*. Using the same protocol for seeds of P. *berteroniana* as the *P. santossi* we obtained very effective control of contamination of plant material. The mortality rate after sterilization in our plant was only 0–2%.

For other members of Bromeliaceae family, were applied more intensive method of sterilization in comparison with our type of surface sterilization. Sterilization by NaClO was useful also for other bromeliads such as *Vriesea reitzii* 70% ethanol for 3 min and 2% NaClO for 35 min were applied (Alves *et al.*, 2006).

The seeds of *Dyckia distachya* (Bromeliaceae) were immersed in 70% ethanol for 3 min under aseptic conditions, and then in a 5% NaClO solution for 30 min (Pompelli and Guerra, 2005). All these methods of surface sterilization provide small mortality ration from 0 to max 10% of disinfected plant material, same as in our study of *P. berteroniana*.

It is possible to use the lower concentration of NaClO for surface sterilization like in *Aechmea fasciata* (Bromeliaceae) (Vinterhalter and Vinterhalter, 1994; Huang *et al.*, 2010) where the florets were successfully surface-disinfected with 0.5 % NaClO for 15 minutes (Huang *et al.*, 2010). The ratio of contamination mortality ranged only from 0 to 5%.

6.2. *In vitro* propagation

Determination of the appropriate medium for the multiplication of explants is the basic indicator of economic frugality method in micropropagation (Knitl, 2011). Micropropagation offers a rapid means of production of clonal plants for conservation and commercial purposes (Bonga, 1982).

Mercier and Kerbauy (1997) optimized systems of micropropagation in many bromeliads and they indicated as the most important PGRs: BAP and NAA. With this conclusion agree also Arrabal *et al.* (2002), who stated that the several combinations of BAP and NAA induce *in vitro* regenerations in bromeliads, i.e. in *Crypthantus sinuosus*. These types of plant growth regulators are very effective for providing adventitious buds or axillary branching. On the other side, Karp (1995) and Hirimburegama and Wijesinghe (1992) declared that the general addition of plant growth regulators can cause the inhibition of shoot multiplication or promote undesirable responses. Nevertheless, for *P. berteroniana* this argument was not confirmed.

Pedroza-Manrique and Tibocha (2008) reported an efficient *in vitro* propagation protocol for *Puya santossi*. They tested the effect of five concentrations of IBA and BAP. The medium supplemeted with BAP at concentation 0.5 mg.l^{-1} produced in average 1.85 ± 0.63 shoots per explant. This treatment in our experiment provided 2.88 ± 0.49 shoots per explant. This concentration of BAP was the only common for both experiments. In *Puya santossi* the best results were reported for medium enriched by 1.5 mg.l^{-1} BAP, which is different in comparison with our study on *P. berteroniana.* For species under our study, the highest number of adventitious offshoots was provided by medium 0.1 mg.l^{-1} BAP.

Other treatments provided the plants with smaller height, lower proliferation and regeneration rate. Thus, it can be concluded that BAP is appropriate cytokine for multiplication programs (Rodríguéz *et al.*, 2003), but optimal concentration is genus specific.

Galvanesi *et al.* (2007) obtained trought the direct morphogenesis in *Aechmea distichantha* an average mean shoot at maximum 138.71 on liquid medium with 5.0 mg l^{-1} NAA and 5.0 mg.l^{-1} BAP, supporting the positive effect of BAP and NAA on propagation. Similarly, Mendes *et al.* (2007) obtained 5.1 and 5.3 shoots per stem in concentration 0.3 mg.l^{-1} NAA + 1.1 mg.l^{-1} BAP and 0.5 mg.l^{-1} NAA + 1.1 mg.l^{-1} BAP, respectively. Similar results were obtained by (Arrabal *et al.*, 2002) in *Crypthantus sinuosus*. These studies clearly show that combination of BAP and NAA can be beneficial for propagation of some bromeliads. However, in our case addition of NAA decreased formation of adventitious shoots.

Surprisingly, zeatin did not provide satisfactory results in our experiment. In many species from different families, e.g. Ericaceae or Orchideaceae the multiplication of shoots was successful using of zeatin (Giridhar and Ravishankar, 2003; Debnath, 2005; Cüce *et al.*, 2013; Sedlák and Paprštein, 2011).

The number of shoot tips of *Vanilla planifolia* from family Orchideaceae was maximal on medium with 0.5 mg.l^{-1} zeatin (Giridhar and Ravishankar, 2003). Debnath (2005) reported in *Vaccinium vitis-idaea* L. belonging to the family Ericaceae, that the highest production of shoots per explant can be achieved on medium containing 0.2 mg.l^{-1} zeatin. Similarly, in *Vaccinium macrocarpon* the highest amount of shoot was achieved on meidum supplemented by 1.0 mg.l^{-1} zeatin (Sedlák and Paprštein, 2011).

6.3. *In vitro* rooting and *ex vitro* tranfer

Various autors reported the influence of NAA on root formation in several bromeliads. Mercier and Kerbauy (1995) and Droste *et al.* (2005) stated that the most appropriate auxine for *in vitro* rooting formation is NAA.

García *et al.* (2009) also used NAA for root formation in *Neoglaziovia variegate* and recommended the concentration 0.1 mg.l^{-1} NAA. *In vitro* rooting of *Hohenbergia penduliflora* (Peréz *et al.,* 2013) was mainly influenced by NAA in concentration 0.6 mg.l^{-1}. Using this concentration, the highest percentage of roots formation per explants was achieved.

In our experiment, we found that intermediate concentration of NAA (i.e. 0.3 mg.l^{-1}) is optimal; lower and higher concentrations of NAA decrease formation of roots.

The use of IAA for root formation in *P. berteroniana* did not show satisfactory results, regardless of concentration used. However, in other species from various families, IAA showed better results than the use of NAA. Rooting of mature clones in *Acacia mangium* belonging to the family Fabaceae, showed the best results on medium enriched by 0.7 mg.l^{-1} and 1.0 mg.l^{-1} IAA (Monteuuis and Claude Bon, 2000). Debnath *et al.* (2000) and Bhuiyan *et al.* (2011) reported similar results with lower concentration of IAA in *Colocasia esculenta* from the family Araceae.

For *ex vitro* transplantation of *Crypthantus sinuosus* well rooted plants higher than 1 cm were used (Arrabal *et al.,* 2002). The survival ratio of explants was similar like in *P. berteroniana*. These explants were transferred into the agriculture substrate mixed with sand (1:1). This type of substrate was also used for *P. berteroniana* but instead of sand the perlite was used.

Peréz *et al.* (2013) reported for *Hohenbergia penduliflora* (Bromeliaceae) the most appropriate substrate for transplanting is 100% filter-cake-sugarcane ashes. The percentage of survival explants reached up to 100% after one month. These results indicate that already formed roots are functional even after *ex vitro* transfer, and/or the plants are able to produce new roots in the substrate.

6.4. Flow cytometry

Ploidy level of *in vitro* plantlets of *Puya berteroniana* after *in vitro* propagation was stable. Similar results in *P. berteroniana*, were reported for various plants derived via direct morphogenesis.

Where the flow cytometry was employed and ploidy level homogeneity was found in regenerants, i.e. in cotton *Gossypium hirsutum* (Jin *et al.,* 2008) and *Gentiana panonnica* (Fiuk *et al.,* 2010).

The ploidy level stability was found also in the *in vitro* derived plants of oil palm *Elaeis guineensis* (Rival *et al.,* 1997) and grapevine *Vitis vinifera* (Yang *et al.,* 2008).

On the contrary, the ploidy variations were detected in plants obtained trough somatic embryogenesis in *Picea abies* (Fourré *et al.,* 1997), *Cucumis sativus* (Kubaláková *et al.*, 1996) and *Cyclamen persicum* (Borchert *et al.,* 2007) after *in vitro* propagation.

Flow cytometry is an efficient method for routine large-scale studies of ploidy level in many plants. Method of flow cytometry is appropriate for many plants thanks to its rapidity, reliability and cheapness (Orbović *et al.,* 2008).

Flow cytometry has not been used only for ploidy detection in plants after *in vitro* propagation but also for naturally occuring plants, e.g. in *Puya raimondii* genome size was detected as 1.13 pg (Sgorbati *et al.,* 2004). The chromose number of *P. raimondii* was detect by McWilliams (1974) as 2n = 2x = 50.

This type of analysis is very beneficial, as it may be used in furter for the breeding effort in the genus *Puya*.

6.5. Extraction of DNA

Genomic DNA of bromeliads is mainly extracted from fresh or dried leaves. In various bromeliad species the isolation of DNA was done following of Cetyl trimethylammonium bromide (CTAB) based procedure (Doyle and Doyle, 1987). Extraction of DNA by using of CTAB protocol was used in *Aechmea* genus (Wang et al., 2012), *Alcantarea* genus (Versieux et al., 2012), *Neoregelia* genus, *Guzmania* genus, *Vriesea* genus (Zhang et al., 2012). DNA concentrations of these genuses were estimated in 0.8% agarose gel. This method of DNA extraction is also popular in many other families (Ge et al., 2012; Russel et al., 2010), etc.

Using of classical CTAB procedure, in the genus *Puya* did not provide the sufficient amount and quality of DNA. Similar results were reported by Schulte et al. (2010) who used for Chilan *Puya* species modificated CTAB protocol.

Described modification of CTAB procedure according to Weising et al. (2005), includes the purification step to precipitate remaining polysaccharides (Saghai–Marrrof, 1984). In previous research, commercial ready-to-use DNA extraction kits did not always result in sufficient amount of DNA isolates causing various problems in the restriction in subsequent PCR amplification, they were therefore not further used (Schulte *et al.,* 2010).

Sgorbati et al. (2004) isolated the genomic DNA from leaf samples from of *P. raimondii*. Genomic DNA was performed in 0.5 g of fresh plant material, but for extraction of *P. berteroniana* was used only 100 – 120 mg of plant material. In study of *P. raimondii* samples were processed by CTAB buffer and the DNA was purified by using of α - amylase and RNAse A. On the other hand Hornung – Leoni et al. (2013) extracted the DNA from silica gel dried leaf tissue. Samples were frozen in liquid nitrogen similar like in *P. berteroniana* with addition of mercaptoethanol and ground to a powder.

For the extraction, it was used CTAB method modified by using of RNase and incubated for 30 min to eliminate RNA in the samples. DNA concentration was adjusted to 200 ng per µL for each sample. Without the using of RNAse was obtained the sufficient amout of DNA for experiments.

6.6. ISSR analysis

ISSR markers are consided to be reliable in detection of somaclonal variation or genetic stability in plants after micropropagation. In our experiment, ISSR markers did not reveal any genetic changes suggesting that somaclonal variation in regenerants after *in vitro* propagation. Similarly, Lata *et al.* (2010) applied ISSR markers in *Cannabis sativa in vitro* plants, where genetic fidelity was detected. *In vitro* regenerants of *Ochreinauclea missionis* (Rubiaceae) multiplied by nodal segments were also tested for genetic stability using 32 ISSR primers. However, a total polymorphism 2.73% was found (Chandrika and Ravisharkan, 2009). Huang *et al.* (2009) applied the ISSR markers for detecton of genetic stability in regenerants of *Platanus acerifolia* from Platanaceae family. Screening of 38 ISSR primers provided 16.5% total polymorphism indicating that genetic fidelity was not maintained.

Use of other molecular markers or their combination can be also beneficial for detection of genetic stability of variability in regenerants of plants from various families, as reported for; *Centaurea ultreiae* (Mallón et al., 2010); *Pissum sativum* (Smýkal et al., 2007); *Phoenix dactylifera* (Saker et al., 2006) and *Elaeis guineensis* (Matthes et al., 2001).

So far, in family Bromeliaceae, only RAPD has been applied to reveal somaclonal variation after *in vitro* propagation.

Santos *et al.* (2008) evaluated genetic variability in plantlets of *Ananas comosus* var. *bracteatus* using RAPD markers. In total, 2.8% polymorphism was detected.

In our study, the use of ISSR seems to be sufficiently reliable and it can be recommended also for other scientific studies associated to genetic variability in *Puya berteroniana.*

7. Conclusion

The *in vitro* culture of *Puya berteroniana* was successfully established using 70% ethanol and 1% NaClO.

For *in vitro* propagation, 16 different combinations of PGRs at various concentrations were used. As the most appropriate medium for *in vitro* propagation of *P. berteroniana* was ½MS medium containing 0.1 mg l^{-1} of BAP providing approximately 5.46 ± 0.91 of new shoots per explant.

Explants of *Puya berteroniana* successfully rooted on different media. The most successful treatment was ½MS supplemented by 0.3 mg l^{-1} of NAA. In this treatment, plants created approximately 10.0 ± 0.95 roots and the percentage of survival plant after transferring into the *ex vitro* conditions was high (99 %).

To evaluate the effect of regeneration on ploidy stability, the flow cytometry analysis was applied. Results indicated that plants of *Puya berteroniana* obtained by direct morhpogenesis are not very susceptible to alteration of ploidy level.

Genetic stability was verified in 11 plants propagated via direct morphogenesis in order to exclude occurrence of somaclonal variation. The amplified products from 6 ISSR primers were monomorphic accross the plants and were identical to the mother plant.

Optimized protocol for micropropagation of *P. berteroniana* can be used for large-scale propagation of this species

8. Recommendation

As the most problematic part of the thesis was the extraction of genomic DNA from the leaf tissue. The use of commercially available purification kit should be tested in order to obtain higher concentration of genomic DNA.

ISSR markers have been in this study optimized for species *P. berteroniana*. Application of other molecular markers should be also tested to confirm the results.

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