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Faculty of Tropical AgriSciences

Use of *in vitro* cultures in *Incarvillea delavayi*

Bachelor's thesis

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Declaration:

I declare that I have written this Bachelor thesis "Use of *in vitro* cultures in *Incarvillea delavayi*" independently and cited from official resources which are written at the end of this work.

Prague 2.4.2016

Tomáš Hovorka

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List of Abbreviations

ANOVA	Analysis of variance
BAP	6-benzylaminopurine
B5	Gamborg B5 (1968) medium
2,4-D	2,4-dichlorophenoxyacetic acid
FTA	Faculty of Tropical AgriSciences
GA_3	Gibberellic acid
IAA	Indole-3-acetic acid
2iP	Isopentil adenin
KIN	Kinetin
LS	Linsmaier and Skoog (1965) medium
MS	Murashige and Skoog (1962) medium
NAA	1-naphtalenacetic acid
PGR	Plant Growth Regulator
S.D.	Standard deviation
SH	Shenk and Hildebrant (1972) medium
TDZ	Thidiazuron
USA	United States of America
ZEA	Zeatin

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Abstract

Incarvillea delavayi (Bignoniaceae) is a perennial herb with attractive pink flowers and it is used especially for ornamental purposes. The aim of this thesis was to optimize process of *in vitro* propagation in this ornamental species. As an initial plant material, seeds obtained through Index Seminum program were used. Two genotypes originated in Bratislava Botanical Garden (Slovakia), and one in Saint Galen Botanischer Garten (Switzerland) were used for the experiment. Seeds were surface sterilized using 70% ethanol and 1% NaClO than cultivated on ½ MS (Murashige a Skoog, 1962) medium until they germinated. For initial multiplication of plant material, the plants were repeatedly sub-cultivated on ½ MS medium with addition of cytokinin 6-benzylaminopurine (BAP) at concentration of 0.5 $mg.l^{-1}$. For *in vitro* propagation experiment, BAP at concentrations 0.1 $mg.l^{-1}$, 0.5 $mg.l^{-1}$ and 1.0 $mg.l^{-1}$ were tested. Medium (½ MS medium) without the addition of PGRs was used as a control. Influence of genotype, BAP and influence of both concentration on shoot production was also tested. Root induction was tested using two concentrations (0.1 $mg.l^{-1}$ and 0.3 $mg.l^{-1}$) of 1-naphtalenacetic acid (NAA) in ½ MS medium. Results clearly showed that BAP and NAA at higher concentrations have significant influence on production of shoots and roots under *in vitro* conditions. Optimal shoot production (8.9 ± 2.68 shoot per explant) was achieved on ½ MS medium with addition of 1.0 $mg.l^{-1}$ BAP. Genotype and combination of genotype and BAP concentration have not significantly affected shoot production. The highest number of roots (9.8±2.64 root per explant) as well as their length (8.8±4.14) cm were achieved on medium supplemented with 0.3 $mg.l^{-1}$ NAA. In this study, protocol for *in vitro* propagation and rooting in *I. delavayi* were optimized. The protocol might be used for large scale production in this species.

Keywords: adventitious shoots · *Incarvillea delavayi* · *in vitro* propagation · plant growth regulators · rooting

Abstrakt

Incarvillea delavayi (*Bignoniaceae*) je vytrvalá okrasná bylina s velkými atraktivními květy. Cílem této práce byla optimalizace procesu mikropropagace této okrasné rostliny s využitím růstových regulátorů rostlin. Semena získaná prostřednictvím programu Index Seminum byla použita pro založení *in vitro* kultury. Byly použity dva genotypy z Botanické zahrady v Bratislavě (Slovensko) a jeden genotyp z Botanické zahrady v Saint Galen (Švýcarsko). Semena byla sterilizována pomocí 70% etanolu a 1% NaClO a kultivována na ½ MS (Murashige a Skoog, 1962) médiu. Po jejich vyklíčení byly rostliny dále pěstovány na ½ MS mediu s přídavkem cytokininu 6-benzylaminopurinu (BAP) v koncentraci 0,5 mg.l^{-1} za účelem pomnožení rostlinného materiálu. *In vitro* množení bylo testováno pomocí cytokininu BAP o koncentracích 0,1 mg.l^{-1}, 0,5 mg.l^{-1} a 1,0 mg.l^{-1} na ½ MS médiu. Jako kontrolní varianta bylo použito ½ MS médium bez přídavku růstových regulátorů. Rovněž byl testován vliv genotypu a vliv genotypu spolu s koncentrací růstového regulátoru BAP na *in vitro* množení *I. delavayi*. Pro zakořeňování výhonů bylo testováno ½ MS média s přídavkem 1-naftyloctové kyseliny (NAA) ve dvou koncentracích (0,1 mg.l^{-1} a 0,3 mg.l^{-1}). Výsledky prokázaly významný vliv růstových regulátorů BAP a NAA použitých ve vyšších koncentracích na tvorbu odnoží a zakořeňování. Nejvíce odnoží vytvořily rostliny pěstované na ½ MS médiu s přídavkem 1,0 mg.l^{-1} BAP (8,9±2,68 nových odnoží na jeden explantát). Vliv genotypu a vliv genotypu spolu s koncentrací růstového regulátoru BAP na tvorbu odnoží nebyl jednoznačně prokázán. Nejvíce kořenů bylo vytvořeno na médiu s 0,3 mg.l^{-1} NAA (9,8±2,64 na jeden explantát). Tyto kořeny byly také delší (8,8±4,14 cm) v porovnání s kořeny vytvořenými na nižší koncentraci NAA. Protokol optimalizovaný v rámci této studie může být využit k efektivnímu množení druhu *I. delavai* pro okrasné účely.

Klíčová slova: adventivní výhony · *Incarvillea delavayi* · *in vitro množení* · růstové regulátory rostlin · zakořeňování

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1 Introduction

Incarvillea delavayi Bur. Et Franch is a wild perennial herb distributed along the Himalayan mountaines in south-eastern Asia (Fischer *et al*., 2004). The main importance of *I. delavayi* is in the ornamental plant industry. There are four cultivars of *I. delavayi*: ´Pink Bee´, ´Snow Top´, ´Alba´ and ´Bees Pink´ (Grierson, 1961). *Incarvillea delavayi* is also being studied for its chemical constitution, which could be useful due to a high amount of biologically active chemical compounds (Fu *et al*., 2009).

Incarvillea delavayi is mainly propagated by seeds. Propagation can be also mediated by taproots or adventitious shoots. This process is difficult and not effective (Grierson, 1961). Thus, a micropropagation of *I. delavayi* could be beneficial for mass propagation not only of this species in general, but especially of cultivars (Hu *et al*., 2005).

Micropropagation has been successfully realized in other species in Bignoniaceae family and has been shown as an effective way to propagate plant material. However, in *I. delavayi*, micropropagation has never been optimized. Therefore, the aim of this thesis is to optimize an appropriate protocol for micropropagation of this species via adventitious shoots.

2 Literature review

2.1 *Incarvillea delavayi* Bur. Et Franch

2.1.1 Taxonomy

Incarvillea delavayi (Fig. 1 A)Bur. Et Franch belongs to kingdom Plantae, cladus Angiosperms, Eudicots, Asterids, order Lamiales, family Bignoniaceae and genus *Incarvillea* (Grierson, 1961). The name was derived after French botanist Pierre Jean Marie Delavay (Marshal, 2010). Genus *Incarvillea* consists of 16 species, some of the most famous are *Incarvillea forestii, I. sinensisI* (Fig. 1 C)*, I. arguta*, *I. lutea* (Fig. 1 D), *I. compacta* (Fig. 1 E), *I. mairei* (Fig. 1 B), they are often cultivated as ornamental plants (Zhang and Santisuk, 1998). Whole Bignoniaceae family consists of about 116-120 genera and 650-750 species distributed mostly in tropical and subtropical climate regions. Species from Bignoniaceae family often produce large flowers and many of them are widely cultivated as ornamental plants as well as *Incarvillea* (Fischer *et al.,* 2004).

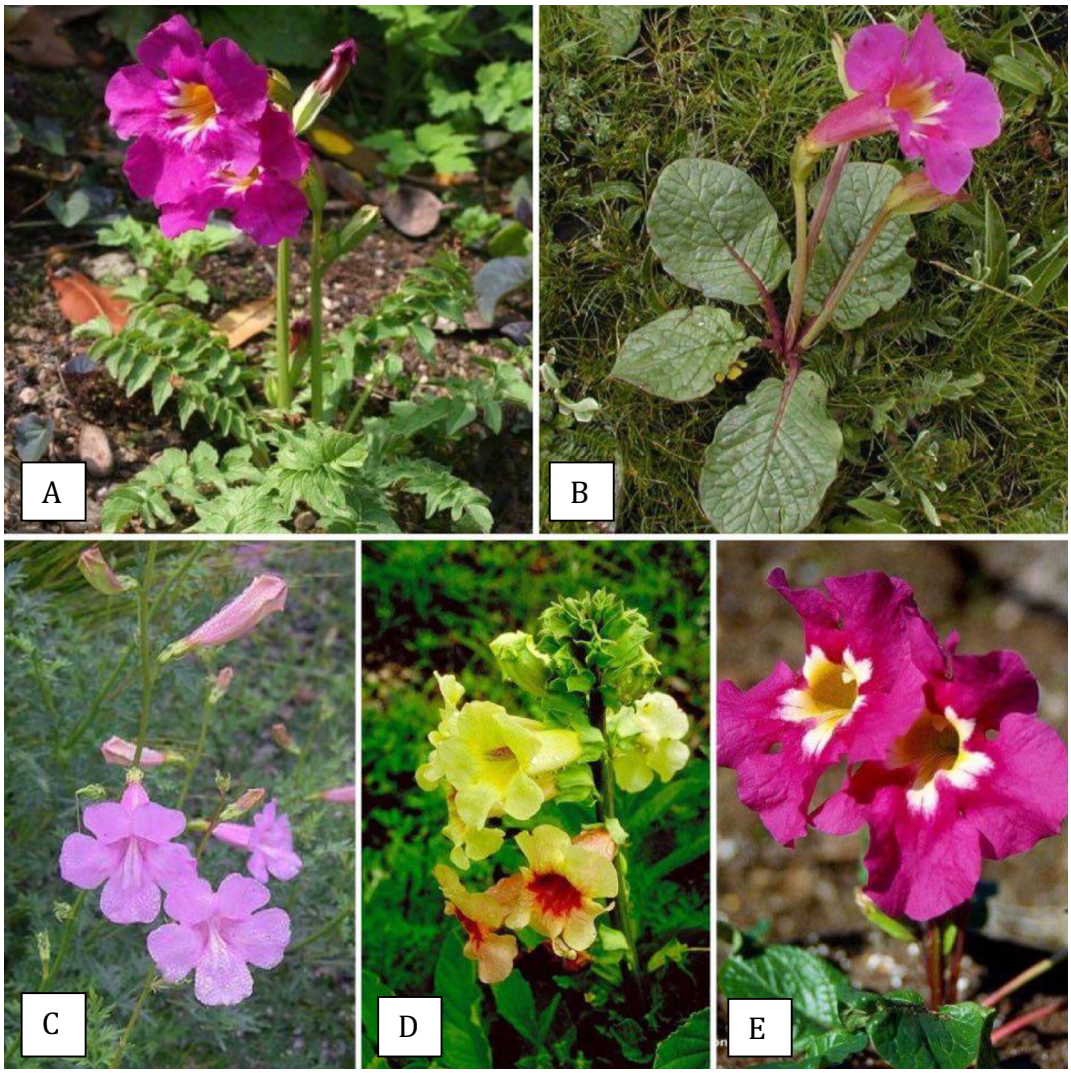

Figure 1. Species from genus *Incarvillea*. (A) *Incarvillea delavayi*. (B) *Incarvillea mairei*. (C) *Incarvillea sinensis*. (D) *Incarvillea lutea*. (E) *Incarvillea compacta*. *(Source: www.zrehacek-alpines.cz (Fig 1A), www.efloras.org (Fig 1B), www.suncrestnurseries.com (Fig. 1C), www.efloras.org (Fig. 1D), www.stewo.no (Fig 1E)*

2.1.2 Origin and distribution

Incarvillea delavayi is a herb (Grierson, 1961), distributed along the Himalayan mountains in Central and Eastern Asia (Fischer *et al*., 2004) (Fig. 2). Most popular is in China, where it is used as ornamental plant for its large flowers (Grierson, 1961). Originally, *I. delavayi* came from Chinese provinces Sichuan and Yunnan. Its natural habitats are Grasslands, slopes, and stony environments among scrubs in altitude from 2400 – 3900 meters above sea level (Zhang and Santisuk, 1998).

Figure 2. Geographical distribution of Incarvillea delavayi indicated by the red circle on the map of East Asia.
(Source: www.beforebc.de)

2.1.3 Morphology

Incarvillea delavayi (Fig. 3 A) is a perennial, stemless, ca. 30 cm tall, glabrous herb. Leaves are basal, 1-pinnately divided, 8-25 cm. Leaf rachises are ca. 20 cm long, lateral leaflets have 4-11 pairs, leaves are elliptic-lnceolate, 4-7 x 1-3 cm in size and they are glabrous. Base is obtuse to subrounded, margin serrate and apex acuminate. Terminal leaflets are 1.5-3.5 x 1-2.5 cm in size, subsessile (Zhang, Santisuk, 1998). Inflorescence is 2-10 flowered raceme, borne on peduncle 15-30 cm long. Pedicels are 0.5-1.5 cm long subtended by linear bract, which is 1 cm long, and bearing 2 smaller bracteoles ca. 0.5 cm long close above the bract. Corolla is 4.5-6 cm long tube, purplish outside on the posterior side, yellow on the anterior and yellow with purple lines on the inside. Lobes varying from mallow purple to cyclamen purple and they are 1.5-2.6 cm long and 1.9-3 cm broad, orbicular or oblate and emarginated at the apex, covered with short stalked glands (Grierson, 1961). Capsule is grey-brown, qudrangular, 5-7.5 cm long and woody. Seeds (Fig. 3 B) are broadly avoid, glabrous on 1 side, pubescend on another and ca. 5 x 3-4 mm (Zhang, Santisuk, 1998). Cotyledons are uncrupled and radical superior (Grierson, 1961).

Figure 3. Plant and seeds of I. delavayi. (A) Above-ground part of *I. delavayi.* (B) Seeds of *I. delavayi*
(Source: www.seedsite.eu (Fig. 2A), www.plantillustrations.org (Fig. 2B)

2.1.4 Reproductive biology

The flowers of *I. delavayi* are borne on a racemose inflorescence that produces 2-10 flowers during season (Grierson, 1961). Inflorescence is indeterminate and it is maturing from the base to the top. Flowers of the genus *Incarvillea* open every day. Flowering is influenced by weather and can last from 1 to 3 days (Han *et al.*, 2008). Species of genus *Incarvillea* have a very specialized structure of flower with obvious herkogamy, usually including a bilobed sensitive stigma and anther appendages (Honglian *et al.*, 2013). *Incarvillea delavayi* is pollinated by insects that are attracted by colour of the flower, and are rewarded by the quantity of honey secreted by a large nectary situated around the base of the ovary. They can be also self-pollinated (Cutting, 1921). Flowers of *I. delavayi,* as well as most species of genus *Incarvillea* have a very special pollination mechanism mediated by insects (Han *et. al.*, 2008). When an insect is passing through the floral tube, it makes a repeated contact with appendages on the anther lobes. The contact with each appendage would result in a separate pollen-shedding event. *Incarvillea delavayi* has an unequal surface of the pollen sac, this has two functions, at first to prevent the pollen sac from being shed all at once and secondly to allow more pollen to be shed directly onto the insects (Cutting, 1921). After pollination results in fruit growth, ligno-coriaceous, quadrancular, curved towards the anterior side and acuminate at the apex capsule. The capsule carries greyish-brown seeds, which are very broadly ovate, long about 5 mm, broad 3-3.75 mm with an entire wing (Grierson, 1961).

2.1.5 Uses

Incarvillea delavayi which is also called “Hardy Gloxinia” is a well-known and popular ornamental plant which is widely cultivated. The natural flower tube is mostly yellow with purple spots, free portions are rose-purplish with purple spots (Cutting, 1921) (Fig. 4 A). Because of the natural beauty of the flower this plant is widely cultivated, the most common with the natural flower is cultivar sold under the name "Bees Pink", whose only difference is in the light pink colour (Grierson, 1961). These cultivars can be found in the shops or in the internet selling more cultivars, which are ´Pink Bee´ (Fig. 4 D), ´Snow Top´ (Fig. 4 C), ´Alba´ (Fig. 4 B). *Incarvillea delavayi* is very adaptable to different garden conditions, and individual plants can perennate for 10 years or even more (Grierson, 1961).

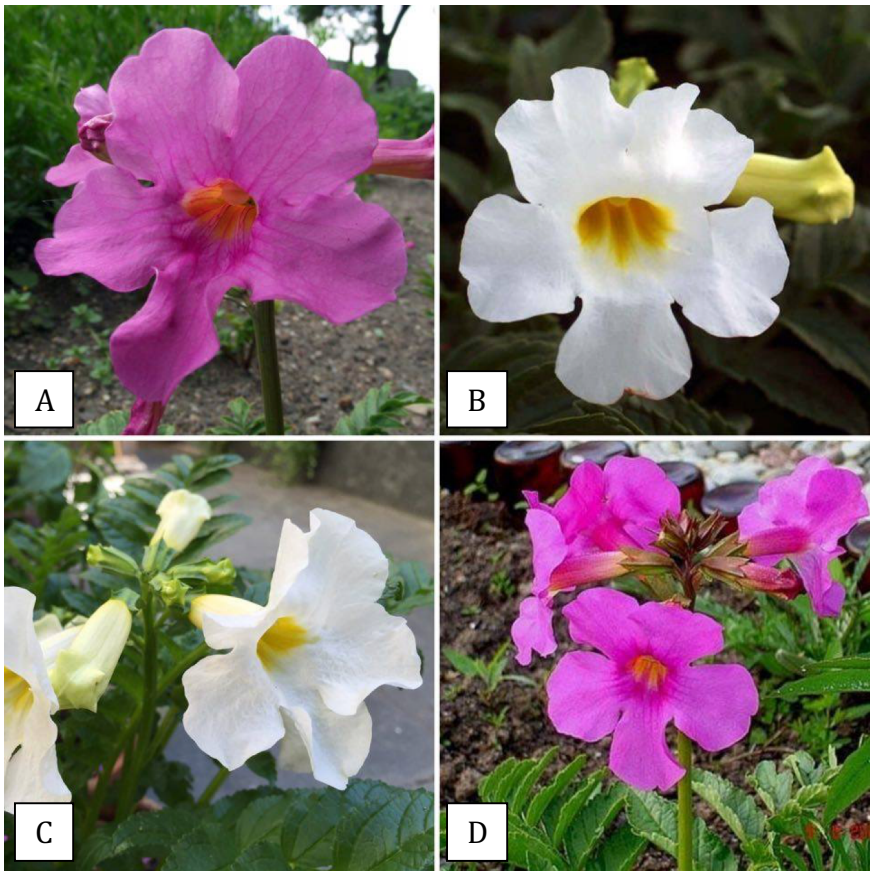

Figure 4. Cultivars of *I. delavay*. (A) Natural flower of *I. delavayi*. (B) ´Snow Top´ cultivar. (C) ´Alba´ cultivar. (D) ´Pink bee´ cultivar.

(Source : www.wikimedia.org, www.prasckac.at, www.frustratedgardener.com, www.willowbrooknurseries.com)

2.1.6 Important chemical compounds isolated from *Incarvillea delavayi*

The most important chemical constituents isolated from plants of genus *Incarvillea* contains alkaloids, ceramides, irinoids, flavonoids, triterpenes etc. Alkaloids are the dominant group within whole genus of *Incarvillea*. Many of those chemical compounds exhibit significant biological activities (Fu *et al*., 2009).

From *I. delavayi* three important compounds were isolated: three new monoterpene alkaloids, delavayine A, delavayine B, delavayine C by Nakamura *et al*., (1999), irinoids 8-epideoxyloganic acid, incarvillic acid by Fu *et al.* (2009), a sesquiterpen named delavayol, oleanolic acid, myrianthic acid and sitoindoside I etc. All of these compounds were isolated from 80% ethanol extract of whole dried plants (Chen *et al.*, 2010). Chemical compounds of *I. delavayi* are now being studied, because in recent years it has been confirmed that irinoids and monoterpene alkaloids from this genus has an antinociceptive and maybe cytotoxical activity (Chen *et al*., 2010).

2.1.7 Plant husbandry

Propagation of plants

Incarvillea delavayi is a perennial herb (Grierson, 1961), but it is usually grown as an annual herb in temperate zones. Plants of *I. delavayi* are often grown from seeds which can be sown directly into the soil in late autumn, or early in the spring. They can be also sown indoors in the growing medium, however, they require temperatures around 15 °C, and light, so they cannot be covered. Germination takes about 25 days. *Incarvillea delavayi* plants can be also grown from taproots, which must be divided from grown plants in spring shortly after flowering.

Soil

Incarvillea delavayi needs moist and well-drained soil, with pH from 6.1 to 7.8. *Incarvillea delavayi* is drought tolerant so it is not necessary for the plant to be regularly watered, natural rain is sufficient.

Spacing

Plants should be grown in a place where they will get full sun in the morning and partial shade in the afternoon. Recommended plant spacing is 30 cm to 38 cm apart (Stephanie, 2013).

Fertilization

Incarvillea delavayi can easily grow without any fertilization, but before planting them into the garden is recommended to put compost into the planting hole. Some gardeners recommend fertilizing them monthly while blooming with balanced fertilizer. Good practice is also to put fertilizer into the soil before planting, however, this should not get directly into contact with the plants of *I. delavayi* so it must be spread around approximately ten square meters of the garden (Stephanie, 2013).

Pests and diseases

A big advantage of *I. delavayi* is that it does not have any serious diseases. The main problem is slugs, so it may be required to protect plants by some required preparation. (Stephanie, 2013).

2.1.8 Use of *in vitro* culture in Bignoniaceae family

Bignoniaceae family represents a big group of mainly ornamental plants across tropical and subtropical regions (Fischer *et al*., 2004). Increasing world population and demand of raw natural material has affected populations of wild plants and some of them are close to extinction, mainly those used in herbal medicine (Prakash *et al.*, 1999). Therefore, it is very important to save these populations or increase their number in the wild, and *in vitro* technologies represent an appropriate method to achieve this. It is also important to ensure enough plant material for ornamental purposes and multiplication of new cultivars. For the ornamental plant industry, *in vitro* cultures especially micropropagation, represent a reliable system of plant mass production. Moreover, micropropagation enables to obtain genetically uniform plantlets. This is very important for genetic stability of new cultivars (Jha, 2005).

Previous studies of *in vitro* techniques in Bignoniaceae family (Tab. 1) using direct or indirect organogenesis have been reported in *Incarvillea sinensis* (Hu *et al.*, 2005), *Catalpa bignoninoides* (Wysokińska and Świątek, 2014) and *Zeyheria montana*

(Cardoso *et al*., 2013). Studies of micropropagation in *Z. montana* and plantlet regeneration in *I. sinensis* have been realized especially due to their strong endemism and difficulty of propagation (*Z. montana*) or endangering of their natural habitat by humans (*I. sinensis*) (Prakash *et al.*, 1999).

As initial plant materials in those studies were used calluses derived from hypocotyls of *C. bignoninoides* (Wysokińska and Świątek, 2014), germinated seeds of *I. sinensis* (Hu *et al.*, 2005) and matured seeds then used for isolation of embryos of *Z. montana* (Cardoso *et al*., 2013).

For cultivation of *I. sinensis*, Hu, Li and Guo (2005) used hormone-free half strength MS medium. Ten different media with addition of various concentration of thidiazuron (TDZ), 6-benzylaminopurine (BAP), kinetin, indole-3-acetic acid (IAA) and 2,4-dichlorophenoxi acetic acid (2,4-D) and their combination for the induction of buds or shoots was tested. Thereafter the most appropriate one was chosen. For rooting ¼ MS was used (Murashige and Skoog, 1962) and a medium with different concentration of IAA, which was the most effective. Plants with well-developed roots were then transferred *ex vitro* for acclimatization, from 100 transferred individuals 76 were transferred to the field condition and grew normally (Hu *et al.*, 2005).

In the remaining two studies of micropropagation in *Zeyheria montana* and *C. bignoninoides* two different media were used. Murashige and skoog (1962) medium with addition of different concentration of BAP and gibberilic acid (GA_3) in six combinations were used in *Z. montana* (Cardoso *et al*., 2013). In *C. bignoninoides* SH (Shenk and Hildebrandt, 1972) medium was used which was the most appropriate. For multiplication process combinations of auxine IAA and cytokinin BAP were tested (Wysokińska and Świątek, 2014). From both studies rooted plants were transferred into the greenhouse condition and grew normally.

Micropropagation in *I. delavayi* has not been studied yet. Based on results of other studies focus on *in vitro* cultures in Bignoniaceae family can be assumed that the optimization of micropropagation of *I. delavayi* might be successful as well.

3 Aims of the thesis

The main objective of this study was to optimize the process of micropropagation in *Incarvillea delavayi* through production of adventitious shoots.
The main objectives were divided in following partial goals:

- Establishment of *in vitro* culture
- Finding an optimal concentrations of BAP for adventitious shoot production.
- Optimization of *in vitro* rooting using NAA at various concentrations.
- *Ex vitro* transfer of rooted plants to greenhouse conditions.

Hypotheses:

- BAP concentration affects the number of leaves and the number of offshoots of the plant regardless of genotype.
- NAA concentration affects the number and length of roots.
- Genotypes differ in response to *in vitro* conditions.

4 Materials and methods

4.1 Plant material

Seeds of *I. delavayi*, obtained through Index Seminum from Bratislava (Slovakia) and Saint Galen (Switzerland) botanical gardens, were used as the initial plant material. Two genotypes, denoted as A and B were obtained in 2014 from Bratislava Botanical Garden, and one genotype, denoted as C in 2015 was obtained from Botanischer Garten Saint Galen.

4.2 Methods

4.2.1 Establishment of *in vitro* culture and multiplication of plant material for the experiment

Establishment of *in vitro* culture took place in the Laboratory of Plant Tissue Culture of the Department of Crop Science and Agroforestry at the Faculty of Tropical AgriSciences (FTA) at the Czech university of Life Sciences Prague in 2015.

Seeds were sterilized by the following process. The seed surface was sterilized using 70% ethanol for one minute. Thereafter, the seeds were disinfected by 2% aqueous solution of NaClO applied for 20 minutes. After that, the seeds were washed twice in sterile distilled water and sown on ½ MS medium supplemented with 100 $mg.l^{-1}$ myo-inositol, 30 $g.l^{-1}$ sucrose and 8 $g.l^{-1}$ agar. pH of the medium was adjusted at 5.7 by KOH. Cultures were maintained in a cultivation box under 16/8 hours light/dark regime at 25/20°C temperature with 36 $\mu mol\ m^{-2}.s^{-1}$ fluorescent light.

Plant rosettes with developed leaves and roots were transplanted on ½ MS medium supplemented with 100 $mg.l^{-1}$ myo-inositol, 30 $g.l^{-1}$ sucrose, 8 $g.l^{-1}$ agar 0.5 $mg.l^{-1}$ BAP and were used for the multiplication of the plant material. For the experiment purposes, plants were repeatedly multiplied on the same medium every 3-4 weeks.

4.2.2 *In vitro* propagation of *Incarvillea delavayi*

Plant rosettes approximately 1 cm high were transplanted on four different ½ MS media supplemented with 0.1 $mg.l^{-1}$ BAP, 0.5 $mg.l^{-1}$ BAP, 1 $mg.l^{-1}$ BAP and 0 $mg.l^{-1}$ BAP as a control. Influence of BAP concentrations and influence between genotypes was evaluated. Each treatment consisted of 10 plants per genotype (i.e. 30 plants per treatment were used).

Cultures were maintained for 28 days under 16/8 hours light/dark regime at 25/20 °C with 36 μmol $m^{-2}.s^{-1}$ cool white fluorescent light. Thereafter, the number of offshoots, leaves per offshoot, height of the whole plant, and number and length of roots was counted.

4.2.3 *In vitro* rooting of *Incarvillea delavayi*

For root induction, ½ MS medium with addition of NAA (1-naphtalenacetic acid) at two concentrations was tested. Plants were transferred on ½ MS medium supplemented with 0.1 $mg.l^{-1}$ NAA and ½ MS medium supplemented with 0.3 $mg.l^{-1}$NAA. Plants were maintained for 28 days under a 16/8 hours light/dark regime days at 25/20°C with 36 μmol $m^{-2}.s^{-1}$ fluorescent light. After that, the number and length of the roots was counted.

4.2.4 *Ex vitro* transfer

Well rooted plantlets were transferred into the garden substrate with addition of perlite in ratio 1:1.

Substrate was sterilized at 100 °C for 2 and ½ hour. Rooted plants were transplanted into sterilized substrate and covered with polyethylene foil to provide humid microclimate. After approximately 14 days, polyethylene foil was removed. *Ex vitro* transfer was realized in the greenhouse of the Botanical gardens of FTA.

4.2.5 Statistical evaluation

In vitro experiments were organized in a randomized design. Statistical evaluation of data was performed by analysis of variance (ANOVA). One-way ANOVA was applied for each factor to evaluate influence of one variable regardless of outcomes of the other. Two-way ANOVA was then applied for evaluation of combined influence of genotype and BAP concentration. LSD post-hoc test of multiple comparison was used wherever equal variability by independent variable could be assumed. In other case the Games-Howell test was applied. Dependent variables (number of roots, length of roots, number of leaves, number of offshoots and height of plant) are described as mean±standard deviation (SD). Data was analyzed using IBM SPSS 20.0.

5 Results

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

For the sterilization of the seeds surface, 70% ethanol and 2% aqueous solution of NaClO was used. This treatment eliminated 90% of contaminations. From ten seeds, nine geminated without any contamination.

Seeds germinated after 7-10 days of cultivation, when first root and cotyledon leaves had been formed (Fig. 5 A). After the next 7 days, more leaves had formed as well (Fig. 5 B).

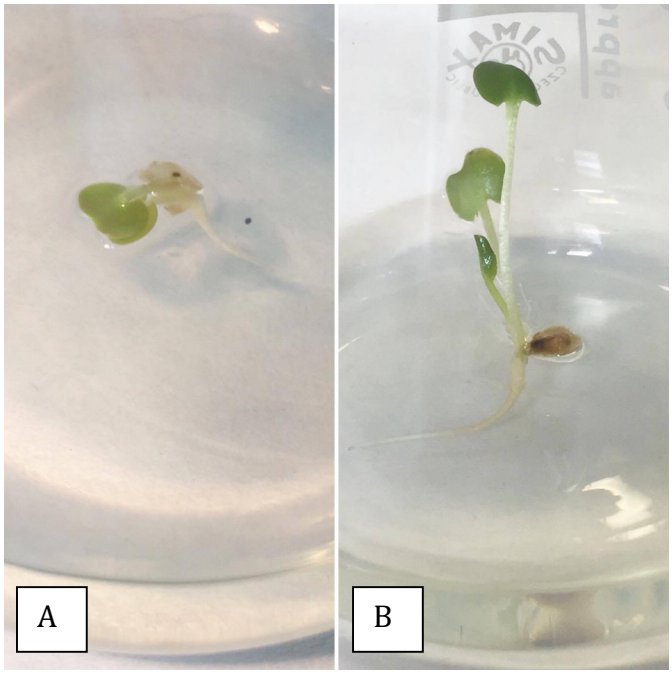

Figure 5. Germinating seeds of I. delavayi 7-10 days after cultivation. (A). First formed root and cotyledon leaves after 7 days of cultivation. (B) Germinated seedling after 14 days with cotyledon leaves and developing true leave.
(Source: author)

From the germinated plants two seedlings were randomly chosen from Bratislava Botanical Garden (genotype A, B) and one from Saint Galen Botanischer Garten (genotype C). The plants were transferred on ½ MS medium supplemented with 0.5 BAP $mg.l^{-1}$and maintained in the cultivation box for ten days. After new shoots were produced, the shoots were separated and cultivated on the medium of the same composition. This process was repeatedly used for multiplication of plant material for the experiments.

5.2 *In vitro* propagation of *Incarvillea delavayi*

As shown in Table 1, the most appropriate medium for shoot production was the treatment ½ MS supplemented with 1.0 BAP $mg.l^{-1}$. The next highest production of offshoots was achieved on the medium with 0.5 $mg.l^{-1}$ BAP. In these treatments the average mean shoot number per explant was 8.9 and 7.7, respectively (see Tab. 1). Plants on medium supplemented with lower concentration of BAP (0.1 $mg.l^{-1}$, 0 $mg.l^{-1}$ BAP) were significantly lower and produced only 3.9 and 2.4 of new offshoots, respectively (see Tab. 1). Higher concentrations of BAP also caused an increase of plant height. There is significant relationship between the BAP concentration and the number of plant offshoots (Fig. 6 A, B, C, D). Higher concentrations of cytokinin BAP provided much better results than lower concentrations.

Table 1. Effect of cytokinin concentration on shoot production

BAP concentration ($mg.l^{-1}$)		Mean shoot number (mean± S.D.)	Height of plant (cm) (mean± S.D.)	Number of leaves per shoot (mean± S.D.)	Number of roots (mean± S.D.)	Length of roots (cm) (mean± S.D.)
0	**a***	2.4±0.72 bcd	3.3±1.36 bcd	3.6±1.07 bcd	0.5±1.11	0.5±1.48
0.1	**b***	3.9±1.14 acd	4.3±1.05 ad	4.3±0.91 ad	0±0**	0±0
0.5	**c***	7.7±2.44 ab	4.9±0.97	4.7±0.83 abd	0±0	0±0
1.0	**d***	8.9±2.68 ab	5.4±1.12	5.7±0.96 abc	0.2±0.91	0.2±1.1

*** Letter in the line behind the number means that there is a significant difference between the number marked with this letter, letters are corresponding with each treatment**

**** Zero means that there were no values measured and values without letters are not significantly different from other values**

The influence of genotype on the medium supplemented with BAP has been evaluated. Statistical analysis showed significant differences in number of roots and height of plants as affected by genotype (see Tab. 2). The number of roots was

significantly higher for plants with genotype K, i.e., 0.4 than for genotypes L 0.1 and M 0, respectively. There was also significant difference found in the height of plants where plants with genotype K were taller than plants with genotype L (see Tab. 2). However, in number of shoots, no statistical differences were found.

Table 2. Influence of genotype on measured factors for all plants regardless on concentration of BAP

Genotype		Number of roots (mean± S.D.)	Height of plant (cm) (mean± S.D.)	Length of roots (cm) (mean± S.D.)	Number of leaves (mean± S.D.)	Mean shoot number (mean± S.D.)
K	**a***	0.4±1.2c	4.9±1.11b	0.3±1.09	4.6±1.06	5.4±3.23
L	**b***	0.1±0.32	4.1±1.43a	0.2±1.19	4.7±1.2	6.5±3.87
M	**c***	0±0a	4.5±1.44	0±0**	4.4±1.34	5.3±2.55

*** Letter in the line behind number means significant difference between number marked with this letter, letters are corresponding with each genotype**

**** Zero means that there were no values measured and values without letters are not significantly different from other values**

The effect of both genotype and concentration of BAP as performed by 2-way ANOVA (LSD post-hoc test) is reported in Tab. 3. The height of the plant is the only characteristic significantly influenced by both genotype and BAP concentration. The effect of genotype and concentration are significant, but there is no significant connection between the two of them and the effect is completely independent (Tab. 3).

Table 3. Effect both of genotype and BAP on measured factors

Genotype	BAP concentration ($mg.l^{-1}$)		Mean shoot number (mean± S.D.)	Height of plant (cm) (mean± S.D.)	Number of leaves (mean± S.D.)	Number of roots (mean± S.D.)	Lenght of roots (cm) (mean± S.D.)
K	**0**	**a***	2.2±0.63cd	4.2±1.23	4.1±1.2 d	1.2±1.62	0.7±1.03
	0.1	**b***	3.8±1.14cd	4.8±0.91	4.1±0.88d	0±0**	0±0
	0.5	**c***	7.5±2.42ab	5.2±1	4.7±0.67	0±0	0±0
	1.0	**d***	8.2±3.16ab	5.5±1	5.4±0.97ab	0.5±1.58	0.6±1.9
L	**0**	**a***	2.5±0.85cd	2.7±1.23cd	3.7±0.95cd	0.2±0.63	0.8±2.37
	0.1	**b***	4±1.33cd	3.9±1.33	4.3±0.67d	0±0	0±0
	0.5	**c***	9.1±2.73ab	4.5±1.08a	4.9±0.99a	0±0	0±0
	1.0	**d***	10.5±2.2ab	5.2±0.94a	5.9±0.99ab	0±0	0±0
M	**0**	**a***	2.5±0.71cd	3±1.23bcd	3±0.82bcd	0±0	0±0
	0.1	**b***	4±1.05cd	4.3±0.71ad	4.4±1.17ad	0±0	0±0
	0.5	**c***	6.4±1.35ab	4.9±0.77a	4.6±0.84a	0±0	0±0
	1.0	**d***	8.1±2.02ab	5.7±1.42ab	5.7±0.95ab	0±0	0±0

*** Letter in the line behind number means signifficant difference between number marked with this letter, letters are corresponding with genotype and treatment**

**** Zero means that there were no values measured and values without letters are not significantly different from other values**

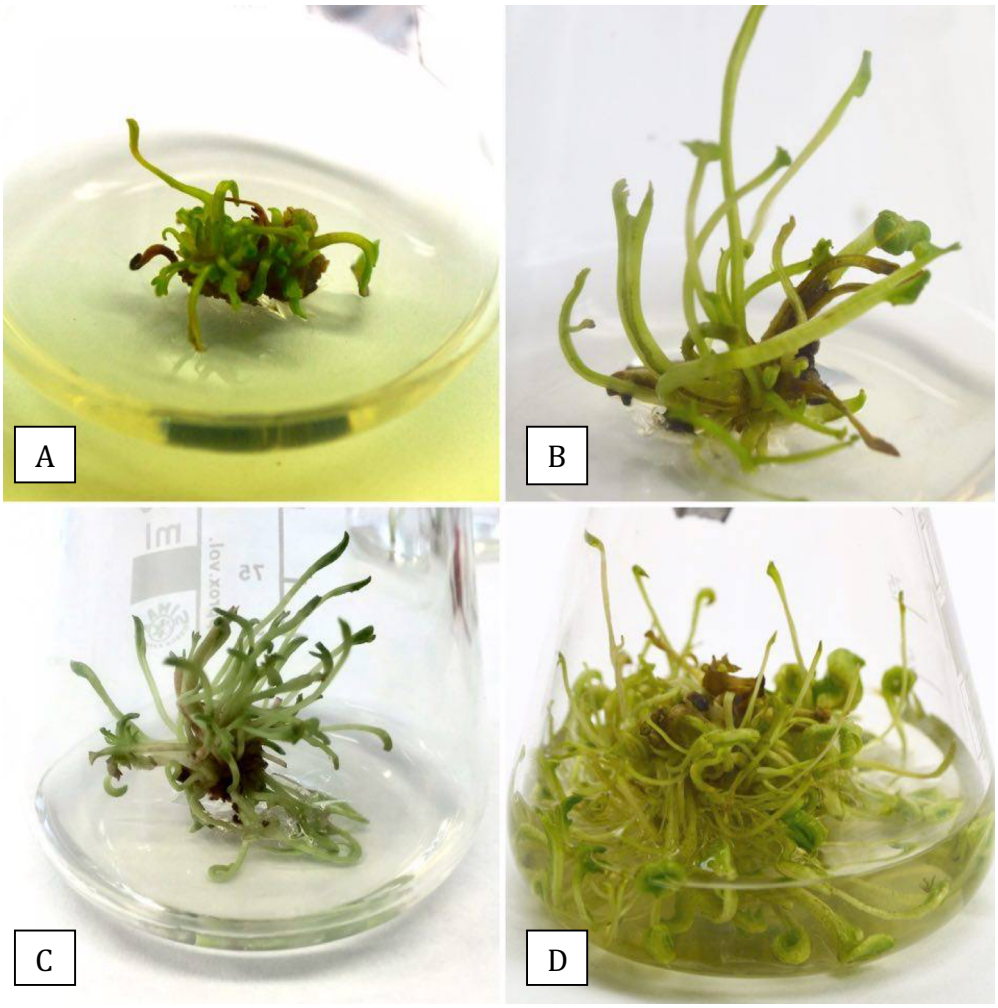

Figure 6. Plants cultivated on ½ MS medium with the addition of BAP. (A) Control plant on BAP free ½ MS medium. (B) Plant on ½ MS medium with 0.1 mg.l^{-1} BAP. (C) Plant on ½ MS medium with 0.5 mg.l^{-1} BAP. (D) Plant pm ½ MS medium with 1.0 mg.l^{-1} BAP. All photos were taken 28 days after cultivation.

Source:Author

5.3 *In vitro* rooting and *ex vitro* transfer of *Incarvillea delavayi*

The most appropriate concentration of auxins for *in vitro* rooting was 0.3 $mg.l^{-1}$ NAA producing 9.8 new roots per explant, 8.8 cm long. Number of roots obtained on ½ MS medium with the addition of 0.1 $mg.l^{-1}$ NAA was rather lower (4.4 roots per explant) and the roots were shorter (see Tab. 4).

After the successful development of roots, the plants were transferred to the *ex vitro* condition (Fig. 7) as described in the methodology section. After 14 days of cultivation, the survival rate was 62.5%.

Table 4. Effect of NAA on *in vitro* rooting

NAA ($mg.l^{-1}$)		Number of roots (mean± S.D.)	Length of root (cm) (mean± S.D.)	Number of offshoots (mean± S.D.)
0	**a**	0.5±1.11 bc	0.5±1.48 bc	2.4±0.72**
0.1	**b**	4.4±3.06 b	5.4±6.51 ac	2.2±0.6
0.3	**c**	9.8±2.64 ab	8.8±4.14 b	2.1±0.68

***Letter in the line behind number means significant difference between number marked with this letter, letters are corresponding with genotype and treatment**

****** **Values without letters are not significantly different from other values**

Figure 7. Plants after *ex vitro* transfer under greenhouse conditions.
Source: Author

6 Discussion

6.1 Sterilization of plant material

The sterilization of the seeds surface and the elimination of bacteria and fungi contamination using 70% ethanol and 2% aqueous solution of NaClO proved to be sufficiently effective as reported also by Cardoso *et al.* (2013), who used the same treatment for *Zeyheria montana*. Bloomfield and Miles (1979) also reported and proved that solution of NaClO is highly efficient against broad range of pathogen organisms. In our case, 90% of thusly treated seeds were without any contamination.

In other studies on Bignoniacease, different chemical agents for sterilization were used. Hu *et al.* (2005) in a study on *Incarvillea sinensis* reported that an effective method of eliminating pathogens is the application of 0.1% solution of $HgCl_2$ for 20 minutes. Malosso *et al.* (2012) reported in a study on micropropagation in *Jacaranda decurrens* higher efficiency with only 3% of contaminated seeds when using 1% Benomyl solution (fungicide) for 1 hour than dipping in 70% ethanol and immersed in $Ca(ClO)_2$ for 30 minutes.

6.2 *In vitro* germination seeds

Hu *et al.* (2005) reported that optimal medium for germination of *Incarvillea sinensis* seeds is ½ MS medium without any PGRs. This procedure was confirmed also by Cardoso *et al.* (2013) who used the same process for seed germination in *Zeyheria montana* from Bignoniaceae family. This treatment was used also in our experiment and the results are congruent with Hu *et al.* (2005) as well as the time period until germination which was 10 days in *I. sinensis* Hu *et al.* (2005) and 7-10 days in this experiment.

6.3 *In vitro* propagation

The selection of an appropriate medium for the multiplication of plant materials using *in vitro* techniques represents a basic affair for economical profitability of the micropropagation process (Knitl, 2011). Micropropagation represents the most important practical and economical application of plant biotechnology and offers a possibility for large scale production of clonal plants, and is also important for conservation of endangered species (Bairu *et al.*, 2011).

The most efficient medium in this study was ½ MS with addition of 1.0 $mg.l^{-1}$ BAP providing high number of adventitious shoots regardless of genotype used. This indicates that this protocol might be probably successfully used for the whole species, as well as for some cultivars of *I. delavayi*.

Hu *et al.* (2005) optimized the process of micropropagation and plantlet regeneration from cotyledon-derived intermediate callus of *Incarvillea sinensis,* which strongly corresponds with the experiment carried out within this thesis as the most appropriate cytokinin was BAP. Hu *et al*. (2005) reported that TDZ (thidiazuron) was less efficient for shoot induction but plants responded as well. In *I. sinensis*, these two cytokinins had the best effect at a concentration of 1.0 $mg.l^{-1}$ or 2.0 $mg.l^{-1}$, respectively, and ensured production of 18.4 shoots per explant, while kinetin or 2,4-D, which were also tested did not lead to formation of shoots. Similarly, Malosso *et al.* (2012) recommended as the most effective PGRs for shoot production from nodal segments, BAP and TDZ at concentration 1.0 $mg.l^{-1}$ which promoted formation of 3.56 buds per stem in *Jacaranda decurrens*. Results of those studies clearly show that cytokinins, especially BAP and TDZ, support or are a critical factor for the initiation of shoots and buds. The results of this thesis are congruent with Hu *et al.* (2005) and Malosso *et al.* (2012).

González-Rodríguez *et al.* (2010) reported an efficient protocol of adventitious shoot induction for *Taeuia donnell-smithii* which belongs to Bignoniaceae family. They tested the effect of six concentrations of BAP, 2iP (isopentyl adenin), KIN (kinetin) and seven concentrations of ZEA (zeatin) on the induction of adventitious shoots from auxilary shoots. The medium supplemented with BAP at a concentration of 1 $mg.l^{-1}$ produced 0.33 shoots per explant, and BAP started to be effective with increasing concentration. The most effective concentration was 3.3 $mg.l^{-1}$ producing 1.12 shoots per explant. In this species using BAP was not effective therefore

application of ZEA was effective and needed for inducing shoot production. In our experiment plants responded on BAP very well already at lower concentration therefore application of ZEA was not needed.

The comparison of previous studies indicates that effect and type of cytokinins could be very specific in each species despite the fact that these species might belong to the same plant family, in this case Bignoniaceae.

In many studies on other species from different plant families, a combination of BAP with other PGRs for multiplication and production of adventitious shoots proved to be very effective, e.g. Asteraceae (Kher *et al.*, 2014), Lamiaceae (Tsegaw and Feyissa, 2014), Bromeliaceae (Mendes *et al.*, 2007).

6.4 *In vitro* rooting and *ex vitro* transfer

Various authors reported an influence of NAA on root production in several species of the Bignoniaceae family. Kumari *et al.* (2012) reported as the most appropriate auxin for *in vitro* rooting in *Tecomella undulata* (Bibgnoniaceae) NAA at concentration 5.0 $mg.l^{-1}$ with average number of 4.2 new roots per explant. Dalal and Rai (2004) also used NAA for *in vitro* root induction in *Oroxylum indicum* (Bignoniaceae) and reported significantly higher number of roots at the concentration 0.5 $mg.l^{-1}$ NAA than in treatment with other auxins like IAA which they used as well.

In our experiment, 0.3 $mg.l^{-1}$ concentration of NAA was found to be more efficient (9.8 per explant) for the formation of roots than lower concentration 0.1 $mg.l^{-1}$ (4.4±3.06 root per explant). Lower concentration of NAA decreases the number and elongation of roots, this statement agrees with studies of Kumari *et al.* (2012) and Dalal and Rai (2004).

7 Conclusion

The *in vitro* culture of *I. delavayi* was successfully established without contamination using 70% ethanol and 1% NaClO.

Using various concentrations of BAP it was found that 1.0 $mg.l^{-1}$ BAP produces the highest number of shoots and that these plants are the tallest when compared to lower concentrations of BAP.

Differences between genotypes and their influence on shoot production have not been proved. Only the height of plant has been affected by genotype. This indicates that the micropropagation protocol optimized in this study could be used for propagation of the species, including some cultivars.

Plants successfully rooted on ½ MS medium with auxin NAA. The strongest positive effect on rooting was at concentration 0.3 $mg.l^{-1}$.

Rooted plants were successfully transferred *ex vitro* using garden soil: perlite (1:1) as a substrate.

Optimized protocol for micropropagation of *I. delavayi* could be used in ornamental plant industry for large scale production of this species.

8 Recommendations

Micropropagation process in *I. delavayi* have been optimized using cytokinin BAP for shoot production and auxin NAA for *in vitro* rooting and the protocol proved to be efficient for three different genotypes.

Application of other types of cytokinins (individually or in combination with auxins should be also tested for *in vitro* propagation of the species.

Similarly for *in vitro* rooting, more types of auxins should be tested.

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