Faculty of Bioscience Engineering

Academic year 2013 - 2014

Induction of adventitious roots of *Pentas lanceolata* and their multiplication in bioreactors

Inductie van adventiefwortels van *Pentas lanceolata* en hun vermeerdering in bioreactoren

Eline Mignon

Promotor: Prof. dr. ir. Stefaan Werbrouck

Co-promotor: Dr. Sobri bin Hussein

Thesis submitted in fulfilment of the requirements for the degree of Master of Science in Biosciences: Agriculture and horticulture

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Preface

I performed most of the research for my thesis in the Agrotechnology and Bioscience Division of Malaysian Nuclear Agency. During my stay in Malaysia I learned a lot of new techniques, which were not practiced during courses, and tasted the real research life. These experiences have been beneficial to me from technical and human point of view and certainly give a higher value to my study.

First and foremost I would like to express my sincere appreciation to my promotor, Prof. dr. ir. Stefaan Werbrouck, and my co-promotor, Dr. Sobri bin Hussein, for their guidance and advice during the practical work and for their help in accomplishing this thesis. My special thanks also go to Dr. Rusli Ibrahim for his guidance during my practical work and sharing his knowledge about root culture with me. I would like to extend my sincere gratitude to all the employees of the Agrotechnology and Bioscience Division of Malaysian Nuclear Agency for their kindness and encouragement. Especially to Muhamad Rozaimi, Nur Atiqah Muhamad Anuar, Nurul Atiqah Yahya, Nurhidayah Sajahan, Aishah Haji Hassan, Hawa Aziz, Mohamad Fikqrirullah Fendy, Alif Aziz, Nurill Yani and Norain Mohd Nor for teaching me all the new techniques and making my stay in Malaysia more pleasant.

Last but not the least, my heartfelt thanks go to my parents for giving me the opportunity to accomplish the research for my thesis in Malaysia and for the support they gave me during my study.

Eline Mignon,
Ghent, January 2014

Abstract

Pentas lanceolata is a member of the genus *Pentas* which is famous for its medicinal properties. *Pentas lanceolata* contains interesting components in its roots (anthraquinones, antiplasmoidal quinones, iridoids and caumarines) and is a continuously flowering ornamental. These properties make it worth to start an adventitious roots culture of *Pentas lanceolata*. In this study, the effects of light, darkness and different concentrations of the auxins IBA, IAA and NAA were tested on adventitious root induction from leaf and root explants. The combination of leaf explants on ½ nitrate MS medium supplemented with 1 mg/L IAA placed in light was found as the best method to induce adventitious roots. A adventitious root culture was started in the air-lift bioreactor. Only results from the inoculation density of 7 g/L in medium supplemented with 2 mg/L IBA were obtained. After 7 weeks, the biomass increased from 14 g to 353 g fresh roots. In the study on shoot regeneration, no shoot meristems were obtained. But the cytokinins TDZ and PA induced more green callus nodules and seemed to be more potent for the induction of shoots. This study reported the first successful adventitious root culture of *Penatas Lanceolata* in the air-lift bioreactor. So it is possible to induce adventitious roots and further multiply them in the air-lift bioreactor. Still further optimization of the parameters auxin concentration, carbon source, carbon concentration and inoculation density need to be accomplished.

Keywords: adventitious root culture; adventitious root induction; air-lift bioreactor*; Pentas lanceolata*; tissue culture

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Abbreviations

BA	N-benzyladenine
CIM	Callus-inducing medium
2,4-D	2,4-dichlorophenoxyacetic acid
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
2iP	N6-2-isopentenyl
LD50	Lethal dose 50%
MeJA	methyl jasmonic acid
mF9R	meta-fluorotopolin
MS	Murashige and Skoog
mTR	meta-topolin riboside
NAA	1-naphthalene acetic acid
PA	phenyl adenine
SIM	Shoot-inducing medium
SM	secondary metabolites
TDZ	thidiazuron

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Introduction

Several industries such as pharmaceutical, cosmetic and food industry use plant-derived secondary metabolites in their production processes. The availability of these compounds depends on the plant harvest, which is frequently unstable and mostly takes a long time. The production depends on the season or developmental stage. Due to this facts, plant tissue culture is a more interesting alternative to produce useful natural derivatives in a faster way to answer nutritional, pharmaceutical and cosmetic needs. The genus *Pentas* is famous in Africa because of its medicinal properties. The medicinal function is caused by the natural occurrence of iridoids, alkaloids and anthraquinones. *Pentas lanceolata* belongs to this genus and is a beautiful continuously flowering ornamental, which value is only being fully recognized recently. The roots and flowers produce interesting secondary metabolites. The purpose of this work is to determine the best protocol to induce adventitious roots of *Pentas lanceolata* and further multiply them in the air-lift bioreactor to produce secondary metabolites. And, if these adventitious roots could be regenerated into shoots in order to obtain a beautiful flowering ornamental plant again.

The research was mainly done at the Agrotechnology and Bioscience Division of Malaysian Nuclear Agency. This institute is specialized in adventitious root culture by using the air-lift bioreactor and the pilot-scale bioreactor. The air-lift bioreactor is used to produce a huge amount of adventitious roots (mainly of *Panax ginseng*) in a short period. Later on the adventitious roots are transferred to the pilot-scale bioreactor for commercial production. To acquire the root culture techniques necessary for this study, a training with *Panax ginseng* was essential. Some techniques for shoot culture were acquired with *Ananas comosus* and adventitious roots culture in the pilot-scale bioreactor was only practiced with *Panax ginseng*. A number of experiments were also performed at the University Ghent (Belgium), at the Laboratory of Applied In Vitro Biotechnology.

In the first part of this work a brief presentation of the Rubiaceae plant family and of *Pentas lanceolata* itself is given. Besides, the multiplication of adventitious roots is presented together with a description of the air-lift and pilot-scale bioreactor. Then the literature about secondary metabolites and their elicitation is reviewed, followed by a description about the temporary immersion systems for shoot cultures. The second part is devoted to the description of the general materials and methods, which includes the procedure for the handling of the different recipients and a description of the *in vitro* initiation and the used medium. This is followed by the experimental part, where every experiment is described, with a short introduction, specific material & methods, results & discussion and a conclusion. First, the experiments on the root induction from leaf explants are presented, followed by the root induction from root explants and the acute gamma irradiation. Thereafter the experiments with liquid medium are given, which include the root cultures in a shake flask, a small bioreactor and an air-lift bioreactor. Lastly the experiments about shoot regeneration and multiplication are presented. A general conclusion about all the obtained results is given in the last part.

1. Literature

1.1. *Pentas lanceolata*

1.1.1. Family Rubiaceae

Pentas lanceolata, known as Egyptian Star Cluster, belongs to the family Rubiaceae. This family consists of 611 genera and 13.143 species, which make it the fourth largest angiosperm family after the Asteraceae, Orchidaceae and Fabaceae. Rubiaceae are widespread except in the Antarctic continent. In temperate regions, the Rubiaceae are represented by only 2 herbaceous genera. The bulk of this family is essentially woody and (sub)tropical with greatest diversity in low- to mid-altitude humid forests (Robbrecht, 2013).

This family has a significant economic value. The best-known plants out of this family are *Coffea arabica* and *Coffea robusta*. Also bark of *Cinchona officinales* is used for extraction of quinine for treatment against malaria and *Psychotria ipecacuanha*, which yields ipecac, used as an emetic.

Due to the natural occurrence of iridoids, indole alkaloids and anthraquinones, various natural products are present in rubiaceous plants and they might lead to the development of new drugs. *Rubia cordifolia, Morinda lucida and Pentanisia prunelloides* are already widely used in African traditional medicines (Bukuru, 2003). Not only the secondary metabolites makes this family important. The genera *Gardenia, Ixora, Pentas, Mussaenda* and *Sherardia* with beautiful ornamental plants make this family also important for horticulture (Davis *et al*., 2009).

1.1.2. Genus *Pentas*

The genus *Pentas* contains 40 species widely distributed throughout tropical Africa, tropical Arabica, Madagascar and Comoro Islands. This genus is commonly used by local people as medicinal plants. The roots of *Pentas longiflora* and *Pentas zanzibarica* are some examples. *Pentas longiflora* can be used as a cure for tapeworm, itchy rashes, pimples, malaria and skin diseases. *Pentas zanzibarica* can be used as a remedy for gonorrhea, syphilis and can be a drastic purgative (Bukuru, 2003).

1.1.3. *Pentas lanceolata*

Pentas lanceolata itself is native from Yemen to East Africa. This species is found as a branched bush or a woody herb and can become 0,5 to 1,3 m high (Bukuru, 2003). The leaves are oval or lanceolate-shaped. The star-shaped flowers occur in wide rounded clusters and may be red, lavender, purple, white or shades of pink (Figure 1). These flowers make *Pentas lanceolata* one of the best butterfly-attracting plants. The flowers bloom all summer long and the plant grows best under full sun with moist and well-drained soil. The seeds are very small with the size is comparable with seeds of basilic.

Figure 1: Flowers of *Pentas lanceolata* (Florida museum of natural history, 2013)

Pentas lanceolata is a very interesting plant due to its following characteristics:

- Ethanolic extract from flowers can heal wounds (Nayak *et al*., 2005)
- The roots produce pharmaceutical active compounds: Anthraquinones, steroidal compounds, iridoids and scopoletine

Fractionation of dichloromethane, ethyl acetate and methanol extracts of the roots by various chromatographic methods, resulted in the isolation of five anthraquinones (rubiadin-1-methyl ether, rubiadin, damnacanthol, lucidin-ω-methyl ether**,** damnacanthol-3-methyl ether) and the glycoside (rubiadin-1-methyl ether-3-*O*-β-primeveroside). The hexane extract afforded the steroid β-stigmasterol (Bukuru, 2003). These components are interesting secondary metabolites produced by the roots. Moreover, it is a beautiful continuously flowering ornamental which medicinal value is only recently fully recognized.

1.2. Root cultures

Organ culture is a general term for cultures in which an organized form of growth can be continuously maintained. Important organ cultures are meristem, shoot (tip) , node, embryo and also the isolated root culture. There are two types of organ culture. Culture of organs which are destined to have only a defined size and shape, like leaves, flowers and fruits, are called determinate organ cultures. Cultures of organs where the growth is potentially unlimited, like apical meristems of roots and non-flowering shoots, are called indeterminate organ cultures. In a root culture, the root is not connected with the shoot anymore. A branched root system may be obtained. The discovery of the fact that roots can be grown apart from shoot tissue was one of the first developments in tissue culture science (George *et al*., 2008). Organ culture differs from callus and cell culture because the organs grow out of a defined meristem. Due to this fact, the plant organs keep their integrity and retain the biochemical potential of the original plant, while callus and cell cultures cause a lack of genetic stability and lose the ability to accumulate secondary products (Charlwood, 1991). For many interesting secondary metabolites, the production in cultured cells is too low for large-scale purposes. The main reason is that the metabolism is controlled in a tissue-specific manner. Tissue de-differentiation results in loss of production capacity (Murthy *et al*, 2008). Therefore organ culture (root, embryo and shoot cultures) seems to be more promising to produce plant-derived compounds (Verpoorte *et al*., 2002).

Different root cultures methods are known. One of them are the Hairy-Root cultures (HRCs) where the soil bacterium *Agrobacterium rhizogenes* (Ri plasmid insertion) is used to induce the formation of new roots, called hairy roots. The company ROOTec designed a mist bioreactor (RMB) for the optimal growth of hairy roots (ROOTec, 2013). The hairy roots are the result of genetic transformation and are attractive for the production of secondary metabolites (Flores *et al.*, 1999). But for the transformation, herbicide and antibiotic resistance genes are used as selection markers. The use of these markers has raised public health concern, especially for the products used for food (Murthy *et al.*, 2008).

Adventitious roots, induced from leaf explants by *in vitro* methods, have shown tremendous potentialities of production of secondary metabolites. They are a natural product and grow vigorously in an auxin supplemented medium. The first successful scale-up process was by Choi *et al*. (2000). Adventitious roots of *Panax ginseng* were grown in 500 L balloon type bubble bioreactor (air-lift bioreactor). The biomass increased more than in the hairy root culture systems reported earlier. By means of the elicitation technique with methyl jasmonic acid (MeJA), the interesting component production (saponins) could be elevated up to 2,5%.

Compared to hairy root culture and field cultivation, the adventitious root culture by means of a bioreactor seems to be a more safe, stable and reliable way for the production of nutraceutically and pharmaceutically important metabolites. Table 1 listed the plants in which adventitious roots have been induced and cultured successfully for the production of secondary metabolited.

Table 1: List of plants in which adventitious roots have been induced and cultured successfully for the production of secondary metabolites (Murthy *et al.*, 2008)

Plant species	Metabolite	Importance
Anthemis nobilis	Geranyl isovalerate	Essential oil, fragrance, anti-inflammatory
Cornus capitata	Tannins	Anti-oxidants
Duboisiamyoporoides D.leichhardtii hybrid	Scopolamine, hyoscyamine	Spamolytic, kydriatic agents
Echinacea purpurea E. angustifolia	Caffeic acid derivatives	Immunostimulant, anti-inflammatory, anti-oxidant
Iris germanica	Irigenin, Iristectorigenin A (Flavonoids)	-
Scopolia parviflora	Hyoacyamine (Alkaloïd)	Anticholinergic activity
Panax ginseng	Ginsenosides (Saponins)	Immunostimulant, anti-inflammatory, anti-oxidant, anti-cancer, anti-fatigue
Panax notoginseng	Saponins	Immunostimulant, anti-cancer
Raphanus staivsus L. cv. Peking Koushin	Anthocyanin	Food colouring
Rhus javanica	Galloylglucoses, riccionidin A (Polyphenols)	Anti-oxidants

1.2.1. Multiplication

Before *in vivo* plant material can be transferred to *in vitro,* it needs to be sterilized. If not, the fungi and bacteria will overgrow the explant. It is important to select suitable parts of the plant and the explants need to be free of diseases. Nodal segments, from herbaceous shoots, with axillary buds are required. The leaves need to be removed, because they are not essential in this stage and could increase risk of contamination. After sterilization, the ends of the explants can be damaged due to the bleach. So it is recommended to cut off the ends on a sterile paper before transferring them in the semi-solid MS medium.

To establish the root culture, young root tips from primary or lateral roots are needed. These roots need to be induced on leaf explants grown out of the initiated nodal segment. Discrete stages are needed for the production of secondary metabolites from adventitious root cultures:

- Induction of adventitious roots out of callus masses on leaves (Figure 2A, B).
- Culture of the adventitious roots in a shake flask in liquid medium (Figure 2C) and later in an air-lift bioreactor (Figure 2D).
- Development of strategies for higher accumulation of metabolites and study of the most suitable medium components and environment parameters for a better biomass production.
- Transfer of adventitious roots to a pilot-scale bioreactor of 1000 L (Figure 2E, F). Also for this stage the best parameters need to be optimized.
- Harvest of the roots after a good increase of biomass (Figure 2G). The way of rinsing and drying is very important.

To obtain a good protocol, the best medium components, phytohormones, elicitation products, temperature, inoculum density, pH, light, oxygen, agitation and carbon source need to be selected. These parameters are very significant for a successful harvest.

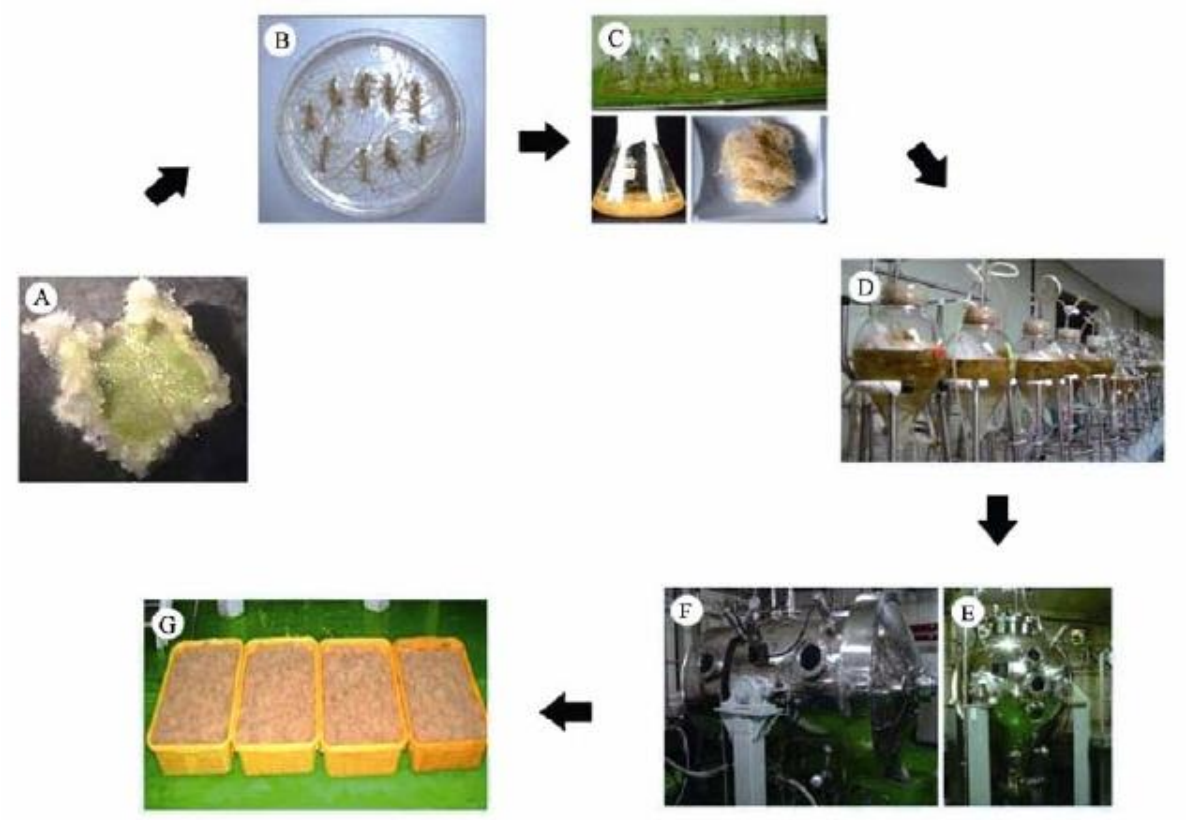

Figure 2: Cultivation of adventitious roots (Murthy *et al*., 2008)

A: Induction of callus out of leaves; B: Induction of adventitious roots from callus masses; C: Shake flask cultures; D: Air-lift bioreactor 20 L; E and F: Pilot-scale bioreactor 1000 L; G: Harvested adventitious roots

1.2.2. Bioreactors used for cultivation of adventitious roots to produce secondary metabolites

'Bioreactor are usually described in a biochemical context as a self-containing, sterile environment which capitalize on liquid nutrient or liquid/air inflow and outflow systems, designed for intensive culture and affording maximal opportunity for monitoring and control over micro environmental conditions.' (Jeong, 2007)

Basic studies of bioreactors for plant cells/organs involve four important practical and scientific issues related to the design and operation:

- Assessment of cell growth and product formation.
- Analysis and modeling of culture dynamics, including integration of biosynthesis and product separation.
- Studies of flow, mixing and mass transfer between the gas, liquid and solid phase, in order to define criteria for bioreactor design and scale up.
- Sterility is a prime design consideration for reactor hardware. (Jeong, 2007)

Air-lift bioreactor (balloon type bubble bioreactor)

There are many considerations for the basic design of the bioreactors. The vessel must be autoclavable, glass seems to be more convenient. Glass allows to observe the cells/organs during cultivation and drops the cost drastically. The lines between air pump/compressor, air filters and air flow meter need to be connected with silicon tubes. The glass sparger is a very important part of the system. They need to produce a constant size of air bubbles. Also the ratio of the diameter and height are important parameters for the basic design of the vessel. Lastly the inoculation and harvest ports need to be suitable for the culture (Jeong, 2007).

The bioreactor systems for plant cell cultures are closely related to microbial culture systems (the vessel design and operating strategies). Many systems are tested for plants and the air-lift (balloon type bubble) bioreactor is considered as the most frequently used type for laboratory-scale cultivation (Figure 3). The shape and liquid mixing are ideal. In the air-lift bioreactor the circulation of the medium and the mixing degree are determined by the gas flow rate. So quite high liquid velocity can be generated without an external recirculation mechanism. The balloon type bubble bioreactor was designed to extend the diameter and to overcome the problem of an increasing height when the volume increases. A 6,5 L bubble column type bioreactor has a height of 100 cm, while a balloon type bubble bioreactor of 5 L is only 32 cm high. Due to the concentric tube part at the bottom the bubbles from the glass sparger lift upwards with a smaller diameter. The smaller air bubbles reduce foam formation and wall growth. Due to its compactness and ability to rapid cell growth, this type of bioreactor seemed to be a model for pilot-scale bioreactor (large-scale) (Jeong, 2007; Lee, 1997).

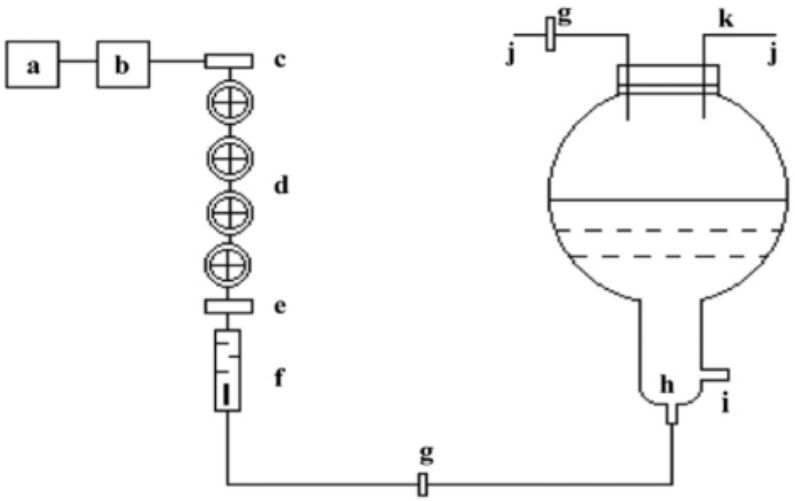

Figure 3: Schematic representation of an air-lift bioreactor (Jeong *et al*., 2009)

The air-lift bioreactor has a 3, 5, 10 or 20 L capacity containing respectively 2, 4, 9 or 19 L liquid medium. a: air compressor; b: air reservoir; c: air cooling device; d: air filter system; e: air dyer; f: air flow meter; g: membrane filter; h: glass sparger; i: medium sampling port; j: vent; k: pre filter

Pilot-scale bioreactor (+1000 L)

Pilot-scale cultivation means that a process has been designed giving a predictable increase in capacity for production of biomass or valuable secondary metabolites. Series of experiments were conducted to set up a large-scale bioreactor system (+1000 L) for the commercial production of *Panax ginseng* adventitious roots. The cultivation consists of two stages. In stage 1 the roots are cultured under optimal conditions to support the root growth, while in stage 2 an elicitor (MeJA) is added to increase the secondary metabolites accumulation (Jeong, 2007).

The pilot-scale bioreactor system consists out of different bioreactor supply systems. Below the different supply systems are described. Further on, the design of the bioreactor vessels and the pipe lines are given.

The water supply system for process water (water used inside the bioreactor) is required for the preparation of the media and for cleaning the bioreactor. Steam, for the sterilization of the bioreactor and pipelines, is generated out of this water. Next to the process water also water for the cooling system is necessary. In particular the process water needs to be of a good quality. To handle contaminants (bacteria, metals, dissolved gasses, ionic charges, etc.), selected treatments must be considered. To purify the water, a distillation system is connected to an ion exchange column. The purified water is stored in the storage tank.

The steam generation system is required because all pipelines, the medium tank and the inside of the bioreactor need to be sterilized before the cultivation. This steam must be free from contamination. To produce high quality steam, the stream generator is connected with the pure water tank.

The air and gas supply system is used for aeration and lifting of the plant cells/organs. Also for transport of the medium from medium thank to bioreactor, compressed air is needed. To prevent contamination like micro-organism, dirty particles, water, etc. from the atmosphere, air filters are installed.

The bioreactor vessel has basically the same design as the previous balloon type bubble bioreactor (air-lift bioreactor) of 20 L. The vessel is built of stainless steel and the inside is polished to have a smooth surface. There are side ports for pH and temperature meters. The sparger is made of compressed stainless particles and produce air bubbles with a diameter less than 0,2 µm. To observe the inside of the bioreactor during the cultivation, a lamp and thick glass are mounted.

The stirred tank reactor (STR) system can be used as medium mixer and as a bioreactor for cell cultivation. This tank is also made of stainless steel.

The medium sterilizer is made of a stainless steel tank and has a double layer to function as an autoclave. The steam can flow between the two layers.

The inoculation bioreactor is a mobile tank which can be moved into the laminar flow. In the laminar flow the roots from the 20 L bioreactor are transferred to the inoculation tank. Further on, the mobile thank is transferred next to the bioreactor vessel and connected with a silicon pipe. True the silicon pipe media flows under air pressure in and out the mobile tank to bring all the roots into the bioreactor vessel. In the mobile tank a blade is installed to cut the inoculated roots.

Pipe lines for water, steam, air and medium are linearly arranged and made of stainless steel.

(Jeong, 2007; oral communication, Hussein 2013)

1.2.3. Medium

Isolated roots are only able to grow *in vitro* in special media. Tissue cultures are commonly maintained in SH medium (Schenk & Hildebrandt, 1972) and MS medium (Murashige & Skoog, 1962). These contain major and minor salts and vitamins together with phytohormones and sucrose. The use of ½ nitrate MS medium advances the root induction (oral communication, Hussein 2013). Roots can be cultured in liquid or semi-solid medium. But liquid media are preferable because on semi-solid medium the salts are less readily available and oxygen availability may be restricted which leads to a slower growth (George *et al*., 2008).

Phythormones

Phytohormones or plant growth regulators are the most important factor. They affect differentiation, cell growth and metabolite formation in tissue culture at very low concentrations. The auxins indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and 1-naphthalene acetic acid (NAA) are often used to induce roots. The effect of these auxins is very genotype dependent. Also their concentration has an influence on the result. For example, IAA was reported to induce adventitious roots in *Antirrhinum majus* (Atkinson *at al*., 1991) and *Helianthus annuus* (Vesperinas, 1998), while IBA is superior over NAA and IAA in the induction of adventitious roots in *Psoralea coryfolia* (Baskaran *et al*., 2009) and *Panax ginseng* (Hahn *et al.*, 2003). For *Eurycoma longifolia,* NAA is more potent in triggering the induction of adventitious roots (Hussein *et al.*, 2012). The different effectiveness of these three different auxins might be affected by the different auxin receptors involved in rooting, the metabolic stability of the auxins and the amount of endogenous auxin. Uptake, transport and conversion of the added auxins are factors who can affect the concentration auxins that reaches the target cells. Conversion is a result of oxidation or conjugation. IBA and IAA are easily irreversibly inactivated by oxidation. But the synthetic auxin NAA might be better for plants with a higher activity of auxin-oxidase. A high concentration of auxins induces a higher level of degrader metabolites in tissue, so they block the regeneration process (Hussein *et al*., 2012).

Carbon source

Root initiation and development processes need a lot of energy. So the carbon sources play an important role as energy sources but also provide the osmoticum (Kubota, 2002). The most commonly found sugar in phloem sap of angiosperms is sucrose and it is easily taken up through the plasma membrane. Therefore in tissue cultures sucrose is the most common carbon source. Dry weight of adventitious roots cultures of *Panax ginseng* increases proportional with an increase of sucrose between 10 to 75 g/L. Although an increasing concentration of sucrose will increase the root growth, the accumulation of secondary metabolites can decrease (Distave, 1998). A higher concentration of sucrose leads to a relatively higher osmotic pressure. Due to this fact, a higher concentration (around 100 g/L) causes a water deficit of the explants and a decrease of dry weight (Jeong, 2007). The effects of sucrose can depend on the plant species. *Eurycoma longifolia* and *Ceratonia siliqua* prefer a concentra-

tion of 50 g/L sucrose, while the best concentration to induce adventitious roots of *Orthosiphon stamineus* was 30 g/L sucrose (Hussein *et al*., 2012).

1.2.4. Other Parameters

Aeration and agitation

The main function of the bioreactor is to transfer oxygen from the gaseous to liquid phase. Aeration gives a better uptake of the nutrients in the medium and causes agitation. Said & Murashige (1979) proved that continuously gentle agitation of tomato roots leads to a doubling of the length growth and an increasing of adventitious root formation. Agitation also prevents the roots from settling to the bottom of the flask. Other purposes are the reduction of plant polarity, the dilution of toxic explant exudates and the uniform distribution of nutrients.

Inoculum density

Inoculum density is an important factor in tissue cultures. Below a critical minimum inoculum density cells fail to grow and it can also affect the secondary metabolite production. For *Panax ginseng* the ideal density is 7 g/L (oral communication, Ibrahim 2013). The minimum inoculum density varies from genotype to genotype and the cultural conditions.

pH

Also pH has an influence on the cell growth and secondary metabolite accumulation, as pH affects the activity of enzymes. A suitable pH is important for the solution of the salts, the uptake of the medium ingredients and the gelling of the agar in semi-solid medium. A lot of plants can tolerate a pH between 4,0 and 7,2 (Butenko *et al*. , 1984). The general pH that is used in plant tissue culture is 5,7-5,8 because of a good gelling of the plant agar at this pH. (George *et al*., 2008). But also the same pH is used for the liquid medium. The pH of liquid medium can fall from 5,7 to 5,17 and even lower during autoclaving, since plant agar has a buffering effect (Singha, 1982). For most species a slightly acid pH seems preferable for adventitious root formation, so the pH fall gives no problems (George *et al*., 2008).

Temperature

The temperature has an impact on several physiological processes. High and low temperatures can cause protein damage. The degree of injury can vary with the plant species, stage of crop development. The optimal temperature lays between 20 °C and 25 °C (Jeong, 2007).

1.3. Secondary metabolites

In the survival strategy of plants, their secondary metabolism always plays a major role. So, secondary metabolites (SM) are not directly involved in the normal reproduction, growth or development of the organism. Usually they provide a defense mechanism against herbivores and diseases agents, attraction of pollinators or seed-dispersing animals, etc. These metabolites are also important for the taste, colour and scent of our food and ornamental plants. During the evolution, plants have evolved a wide variety of secondary metabolites. Terpenoids, alkaloids, anthocyanins, quinones, saponins, flavonoids, lignans and steroids are all extracted from plant material and have commercial applications as insecticides (nicotine, pyrethrin), fragrance (rose oil, lavender oil), drugs, dyes (indigo, shikonin), stimulants (caffeine, nicotine, ephedrine) and flavours (vanillin, capsaisin, mustard oils). So the secondary metabolites are not only interesting for the plant itself, but they are also useful for humans (Verpoorte, 2002; Wink, 2009).

Commonly, several major SM, are accompanied by dozens of minor components. The results are complex mixtures which differ between organs, individual plants and species. These minor compounds are synthesized in tissue-, organ- and developmental-specific way with specific biosynthetic enzymes (Murata *et al.*, 2008). Lipophilic substances are deposed in resin ducts, trichomes, oil cells or in cuticle. The hydrophilic compounds are stored in the vacuoles. Long-distance transport is possible true xylem and phloem if the site of synthesis is not the site of storage.

For years, natural drugs from plants were obtained from tedious and costly extractions. The need of large production fields, specific climate, secondary metabolites production in specific season and the small amount of the active agent in the extract makes the production of natural drugs difficult. The solution for these problems is the plant tissue culture methodology. In this methodology the growth conditions are controlled, the media can be optimized to increase the production op SM and the extraction becomes easier. Selection of best tissue lines is also possible. Bioreactor culture is a step towards the commercial production of SM (Jeong, 2007).

The roots of *Pentas lanceolata* produce pharmaceutical active compounds. Anthraquinones, antiplasmoidal quinones, stigmasterol, iridoid glucoside and scopoletine (caumarine) are the components that are recently found in these roots (Bukuru, 2003; Schripsema *et al*., 2007; Endale *et al*., 2012; Distave, 1998).

1.3.1. Chemical elicitation

Since the biosynthesis of SM is controlled during development of the plant and the SM are accumulated in a response to microbial attack or various stress, it is necessary to enhance the metabolites in the isolated adventitious roots. Elicitation consists of applying physical or chemical stress to plant cells or plant tissue and trigger the accumulation of SM. It can be done by an abiotic elicitors (UV light, pH, heavy metal salts, temperature, etc.), biotic elicitors (pathogenic fungi, chitosan, various protein extracts) and chemical elicitors. These last elicitors, jasmonic acid, methyl jasmonate (MeJA) and salicylic acid (SA), are stress signaling molecules and are frequently used for elicitation. MeJA has been regarded as one of the best elicitors in variety of plant species (Jeong, 2007). The stress signaling metabolites enhance the accumulation of SM but can retard the growth of the adventitious roots, as in adventitious root culture of *Panax ginseng*. Because of this reason MeJA is added two weeks after the roots have sufficiently multiplied (Kim Y. *et al.* , 2005). Chemical elicitation is one of the most successful strategies employed so far. But also irradiation can induce the accumulation of SM, such as anthocyanin pigments. Table 2 gives an overview of plants in which secondary metabolites production is elicited with chemical elicitors.

Table 2: Enhancement of secondary metabolites in the adventitious root cultures by chemical elicitation (Murthy *et al.*, 2008)

Plant species	Elicitors used	Metabolites
Bupleurum kaoi	Methyl jasmonate	Saikosaponin
Hyoscyamus muticus	Methyl jasmonate	Hyoscymine, scopolamine
Panax ginseng	Jasmonic acid, methyl jasmonate, organic germanium, ethephon and methyl jasmonate, methyl jasmonate with auxin	Ginsenoside (Saponin)
Scopolia parviflora	Methyl jasmonate and salicylic acid	Scopolamine

1.3.2. Irradiation

Somaclonal variation is defined as a genetic or epigenetic variation by *in vitro* culture. It can be caused by a point mutation, chromosomal rearrangement or DNA methylation. Although somaclonal mutants gave valuable genetic resources for plant breeding, compared to ionizing radiation and chemical treatments the mutant frequencies are often low. Therefore ionizing radiation is performed in mutation breeding. It can damage DNA, alter sugars and bases, form DNA-DNA and DNA-protein crosslinks and cause single strand and double-strand breaks (Kim *et al.*, 2013). For the induction of plant mutations, irradiation treatment is one of the most common techniques. The mutants out of this treatment are useful for functional studies of genes as well as developing new varieties.

Lee *et al*. reported that radiation can cause a mutation in the gene of the anthocyanin pathway, which gives a variation in the colour of chrysanthemum flowers (Lee *et al*., 2008). Also high levels of amino acids in mutant rice lines were obtained by Kim D. *et al*. (2005). New varieties of crops like cocoa, potato, banana and sugarcane are found (Arnold *et al.*, 1998; Das *et al.*, 2000). As mentioned before, gamma rays (Gy) have the potential to stimulate and enhance secondary metabolites production (Sajahan *et al*., 2013).

1.4. Temporary immersion systems

1.4.1. RITA® bioreactor

The RITA® bioreactor is a temporary immersion bioreactor. Agar medium has been used for micropropagation because of the low cost and the simplicity. But a major disadvantage is the huge amount of labor. A temporary immersion system (TIS) have been designed to reduce handling time together with an increase in the multiplication rates and plant quality. This system usually has a drying and a wetting cycle which is repeated in a given period of time. The TIS system combines the advantages of liquid and semi-solid medium. The plants can grow in an air space and have intermittent total availability of nutrients. The advantages of this system are an increased multiplication, reduced consumables costs, better leaf development, reduced labor costs, reduced hyperhydricity and better prepared plants for hardening (McAlister *et al.*, 2005).

Figure 4 illustrates the design of the RITA® bioreactor. In the culture vessel a raft support system is required to support the shoots. From underneath the culture vessel the liquid medium is pumped with compressed air into the culture vessel. Before the medium drains back to the storage tank, it remains in the culture vessel for a few minutes. The interval period depends on the plant species or on the explants (Gupta *et al*., 2008).

1.4.2. SETIS™ bioreactor

The SETIS™ bioreactor is an improved concept of the TIS. Most disadvantages and problems have been solved. It is easy in handling, compact, secure and gives a maximum surface utilization. Figure 5 illustrates the design and operation procedure of the SETIS™. In this system one medium vessel and one plant vessel are connected. Phase 1 is the stationary phase, all the medium is located in the medium vessel. Phase 2 consists out of the transfer of the medium to the upper culture vessel by supplying compressed air into the medium vessel. After a while the medium returns back to the lower vessel by gravity force, this is phase 3. The last phase is the ventilation phase. To renew the air composition, compressed air is supplied into the culture vessel.

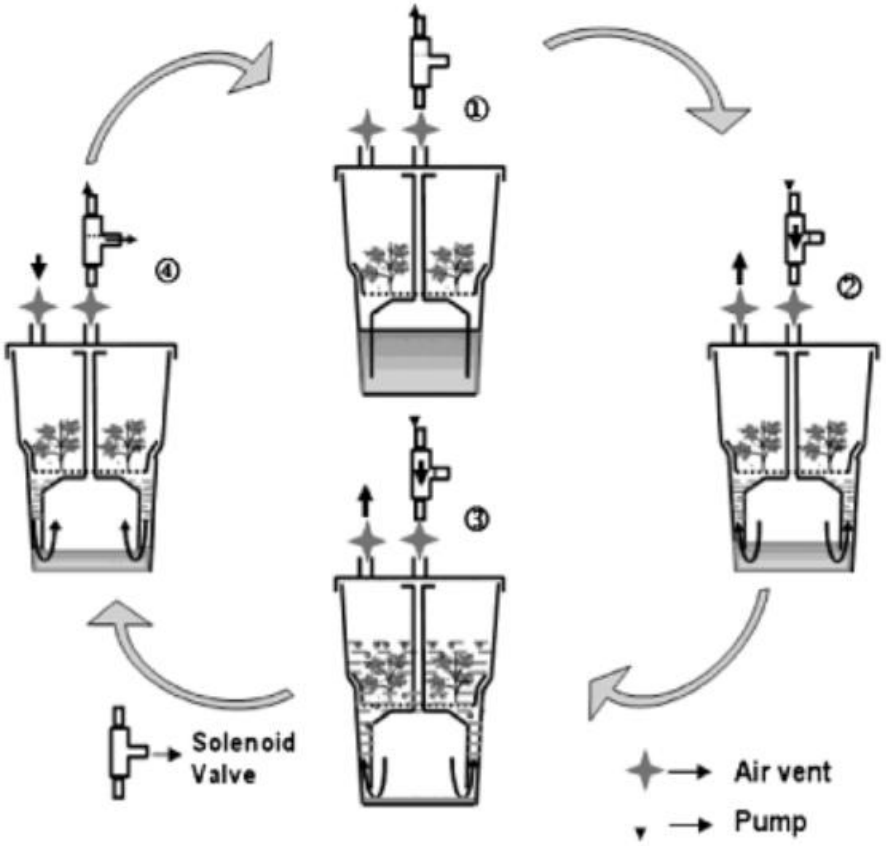

Figure 4: Operation procedure and design of the RITA® bioreactor (Dutta Gupta *et al*., 2008)

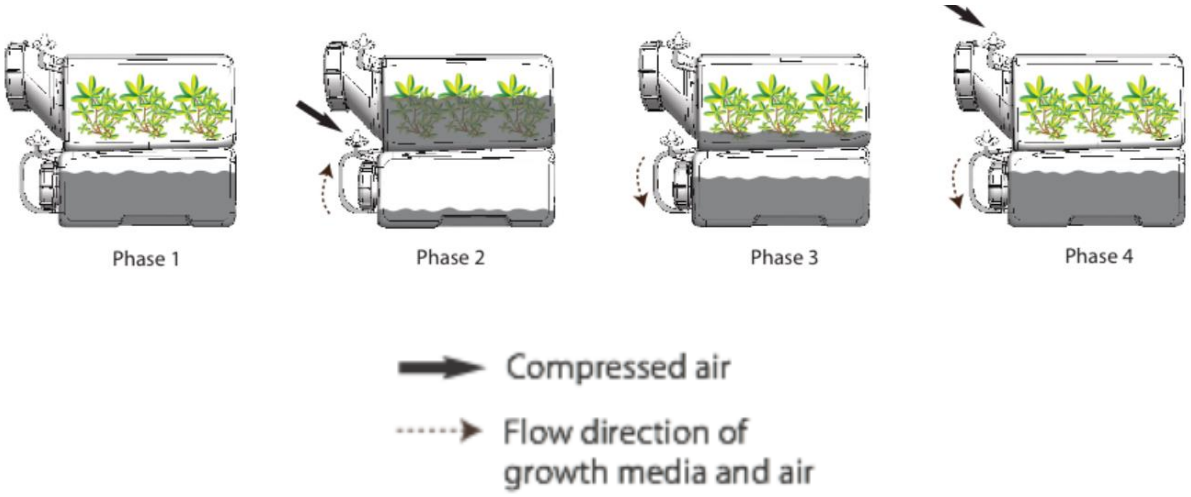

Figure 5: Design and operation procedure of the SETIS™ (Vervit, 2013)

Phase 1: Stationary phase; Phase 2: Immersion phase; Phase 3: End of immersion: Phase 4: Ventilation phase

2. General materials and methods

2.1. Plant materials

This study was performed with *Pentas lanceolata*, more specifically a cultivar with dark pink flowers (Figure 6), grown in the phytotron in the Laboratory of Applied In Vitro Biotechnology of the faculty Bioscience Engineering. This commercially available ornamental plant was selected because of the interesting secondary metabolites produced in the roots as well as its ornamental value.

Figure 6: Start material, *Pentas lanceolata* mother plant with dark pink flowers

2.2. Basic medium

2.2.1. Composition

For all the experiments, the explants were placed in Murashige and Skoog (MS) medium. For the root cultures a ½ nitrate MS medium was used. Table 3 describes the composition of MS medium and in table 4 the total composition of basic MS medium is given. During the experiments the basic medium was supplemented with different concentrations of different phytohormones. The pH was adjusted to 5,8 before autoclaving (15 minutes, 121 °C) and when liquid medium was desired no plant agar/gelrite was added.

Table 3: Composition of Murashige & Skoog medium (Murashige & Skoog, 1962)

Major salts	**mg/L**	**µM**
NH_4NO_3	1650	20600
KNO_3	1900	18800
$CaCl_2.2H_2O$	440	3000
$MgSO_4.7H_20$	370	1500
KH_2PO_4	170	1250
Minor salts	**mg/L**	**µM**
$CoCl_2.6H_2O$	0,025	0,1
$CuSO_4.5H_2O$	0,025	0,1
FeNaEDTA	36,7	0,1
H_3BO_3	6,2	0,1
KI	0,83	5
$MnSO_4.4H_2O$	22,3	0,1
$Na_2MoO_4.2H_2O$	0,25	1
$ZnSO_4.7H_2O$	8,6	30
Vitamins	**mg/L**	**µM**
Glycine	2	26,6
myo-Inositol	100	0,54
Niacin	0,5	4,
Pyridoxine-HCl	0,5	2,4
Thiamine-HCl	0,1	0,3

Table 4: Composition of 1 L basic medium

Component	**Quantity**
Distilled water	1000 ml
MS powder incl. vitamins (Duchefa)	4,405 g
Sucrose (for root cultures)	30 g (50 g)
Plant agar / Gelrite	5 g / 2,3 g

2.2.2. Medium preparation

First all the components of table 4 were dissolved in distilled water, except the plant agar/gelrite. The second step was setting up the pH at 5,8, followed by adding the gelrite. For the preparation of semi-solid medium with agar, the agar had to be dissolved in a small part of the solution. The other part was heated up till it boiled for a few seconds. During boiling, the dissolved agar was added by stirring. Depending on the experiment, the medium was divided over test tubes, bottles or Erlenmeyers. If required, after autoclaving 15 minutes at 121 °C, the medium was poured in the laminar flow in petri dishes.

2.3. *In vitro* initiation

Nodal segments, from herbaceous shoots, of approx. 1 cm with two axillary buds were selected. The present leaves were removed. Figure 7 describes the shape of the explant. Before the transfer to the medium, the selected explants were sterilized. First the explants were rinsed in 70% ethanol followed by soaking for 15 minutes in a mixture of 5% bleach (Twido, 15° NaCl) with three drops of Tween 20. The following steps were done in the laminar flow. After 15 minutes soaking, all the explants were rinsed three times in sterile distilled water. Before transferring the sterilized explants in the semi-solid MS medium, the damaged ends of the explants were removed.

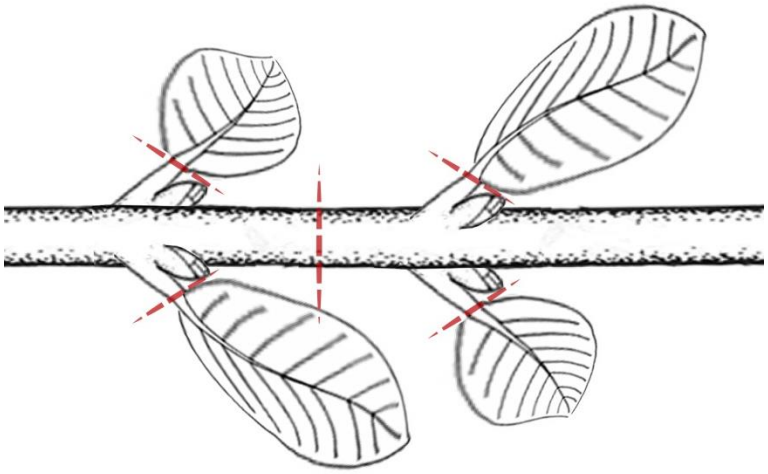

Figure 7: Shape of the shoot explant

red = cutting site

2.4. Micropropagation

After sterilization the nodal segments were transferred under sterile conditions (in laminar flow) to a test tube with semi-solid MS medium. The medium was supplemented with 0,45 mg/L of the cytokinin N-benzyladenine (BA) to produce well growing shoots. Later on, the grown shoots were transferred to a container with medium supplemented with 1,21 mg/L of the cytokinin meta-topolin riboside (mTR) for further propagation. These multiplied *in vitro* plants were used as a stock for the root and leaf explants in the further experiments.

2.5. Transportation

The *in vitro* plant material was carefully transported, in plastic 'Dewit' tubes (Duchefa), with the airplane to Malaysian Nuclear Agency (Figure 8).

Figure 8: Transportation of sterile shoot cultures in plastic 'Dewit' tubes (Duchefa) to Malaysian Nuclear Agency

2.6. Recipients and their handling

2.6.1. Test tube

Each test tube was filled with 20 ml semi-solid medium. After autoclaving and cooling down the medium, the explants (Figure 7) were initiated in the test tubes in the laminar flow and placed in the *in vitro* culture room. To prevent contamination the test tubes were sealed with polyethylene foil.

2.6.2. Petri dish

To prepare a culture in petri dishes the medium was autoclaved in a Duran bottle. After autoclaving, while the medium was still liquid, it was poured in sterile petri dishes in the laminar flow. When the media was totally solidified, the leaf or root explants were initiated directly or the petri dishes were sealed with polyethylene foil and stored for later use. The used petri dishes were also sealed with polyethylene foil and placed in the *in vitro* culture room.

2.6.3. Shake flask

The liquid medium was divided over the Erlenmeyer flasks before autoclaving and covered with aluminum foil. When the sterilized medium was cooled down, the shoots, roots or leaves were transferred in the flasks in the laminar flow. The amount of medium depends on how much shoots, roots or leaves are used. After initiating, the flasks were covered with the aluminum foil and sealed with polyethylene foil. The last step was placing the flasks on a rotary shaker at 100 rpm in the *in vitro* culture room.

2.6.4. Small bioreactor

The classic shake flask system was made of an Erlenmeyer filled with liquid medium and placed on the rotary shaker. When the Erlenmeyer was linked with an air compressor it became a small bioreactor (Figure 9). Because of the air bubbles the rotary shaker was not necessary anymore. The inoculation density remained the same.

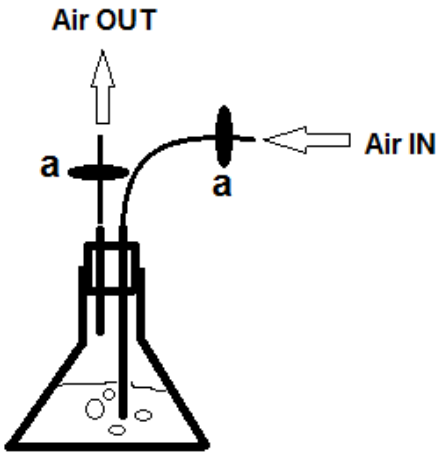

Figure 9: Small bioreactor

a: air filter

2.6.5. Air-lift bioreactor

In Malaysian Nuclear Agency the air-lift bioreactor (Figure 3, 10 and 11) was used to produce a big amount of adventitious roots in a short period, named adventitious roots culture. The adventitious roots were subcultured at intervals of 4 weeks.

Materials

- 3, 5, 10 or 20 L air-lift bioreactor
- Bioreactor rack
- Silicon tubes
- Two 0,2 um membrane filters
- Silicon plugs for inoculation gap
- Silicon plug for falcon centrifuge tube (50 ml, for sampling port)
- Prefilter (nonabsorable cotton)
- Falcon centrifuge tube
- Two aluminum pipes for in the inoculation gap silicon plug
- One aluminum pipe for in the falcon tube silicon plug
- Polyethylene foil
- Autoclave
- Clips
- Laminar flow

- Mobile flame
- Plant material
- Scissors
- Ethanol spray
- Mobile gas flame
- Air pump

Set up bioreactor

Clean all the material with ethanol.

Assembling air line part:

- Cut the silicon tube to about 0,75 m length.
- Connect the silicon tube with the sparger tube at the bottom (Figure 10a).
- Connect the membrane filter (Air IN) with the silicon tube (Figure 10b).
- Connect another piece of silicon tube with the other side of the membrane filter.

Assembling medium line part:

- Cut a silicon tube into 1 m length.
- Connect the silicon tube with the medium tube at the bottom (Figure 10c).
- Connect the aluminum pipe with the other side of the silicon tube.
- Put the aluminum pipe in the middle of the silicon plug of the falcon centrifuge tube (Figure 10d).
- Connect the falcon centrifuge tube with the silicon plug.
- Seal the neck of the falcon centrifuge tube with polyethylene foil.

Plug assembly:

- Put two aluminum pipes into the plug for the inoculation gap (Figure 10e).
- Connect silicon tube of 18 cm with the aluminum pipe.
- Connect this silicon tube with an air filter (Air IN, figure 10f).
- Connect the silicon tube of 38 cm with the other aluminum pipe.
- Fill this silicon tube with the prefilter (non-absorbable cotton, figure 10g).

Closing lid:

- Clean the bioreactor vessel with ethanol.
- Place the plug on the neck (inoculation gap) of the bioreactor.
- Seal the neck op the bioreactor with polyethylene foil.

Sterilization:

- Put the bioreactor inside the autoclave.
- Sterilize for 15 min at 121 °C.
- After sterilization: close the silicon tubes with clips.
- Take the bioreactor out of the autoclave.
- Place the bioreactor in the laminar flow.

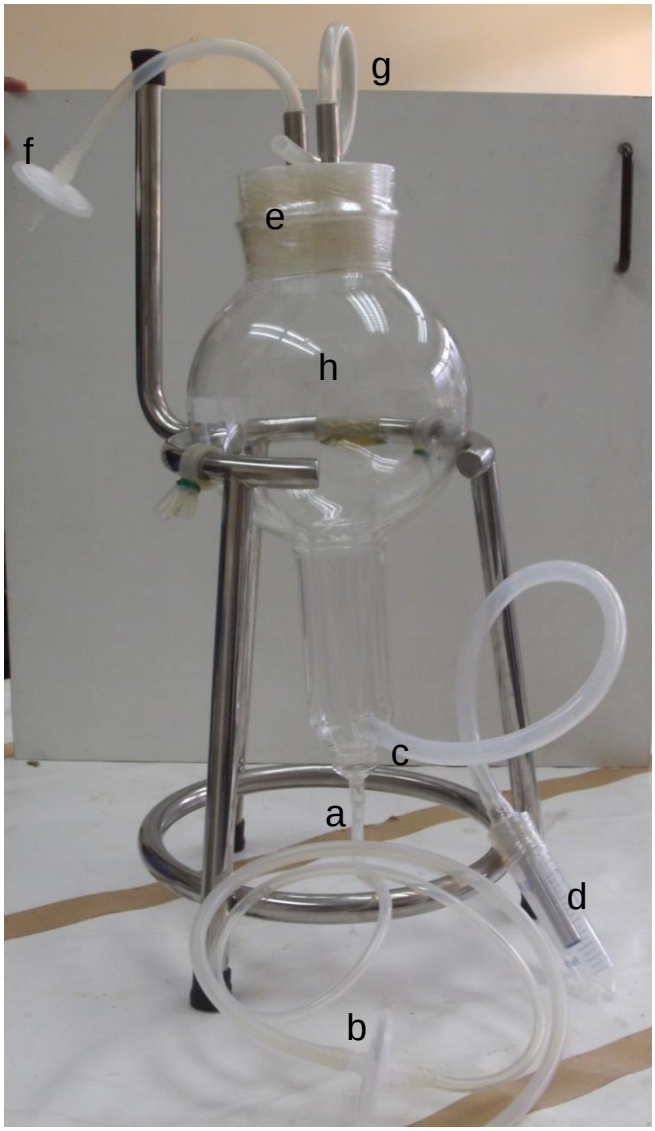

Figure 10: Design of a 3 L air-lift bioreactor before sterilization

a: air line part; b: air filter IN; c: medium line part; d: falcon centrifuge tube; e: plug inoculation gap; f: air filter IN; g: prefilter with cotton; h: bioreactor vessel

Inoculation

Preparation of the explant:

- Select the best adventitious roots (young, no callus, not in aggregated clumps).
- Weigh 7 g/L fresh adventitious roots in a sterile container.
- Flame a scissors.
- Disperse the aggregated adventitious roots.
- Cut the roots into less than 1 cm.

Inoculation of the explant:

- Spray the bioreactor with ethanol.
- Place the bioreactor in the laminar flow with the inoculation gap sloped back.
- Flame the bioreactor vessel with the mobile gas flame till the condense disappears.
- Flame the neck of the inoculation gap.
- Open the inoculation gap.
- Place the silicon plug upside down.
- Flame the neck of the inoculation gap again.
- Pour half of the medium in the bioreactor vessel.
- Flame the neck again.
- Open the container with the explants and flame the neck.
- Bring the explants in the bioreactor vessel with a forcep or by shaking the container.
- Flame the neck.
- Pour the residual medium in the bioreactor vessel.
- Flame the neck.
- Flame the silicon plug.
- Close the inoculation gap with the silicon plug.
- Flame the neck of the inoculation gap.
- Seal the neck of the bioreactor with polyethylene foil.

Connection with air pump:

- Connect the silicon tube of the sparger tube with the air pump (Figure 11a).
- If desirable, this silicon tube can be connected with an air flow meter.
- Remove the clip on this silicon tube after turning on the air pump.
- Remove the clip of the silicon tube with the cotton filter and form a loop with it (Figure 11b).
- Use a clip to close the silicon tube with the air filter (back up for when the electricity goes out, Figure 11c).
- The silicon tube for the medium remains closed (Figure 11d).

(Paek, 2011)

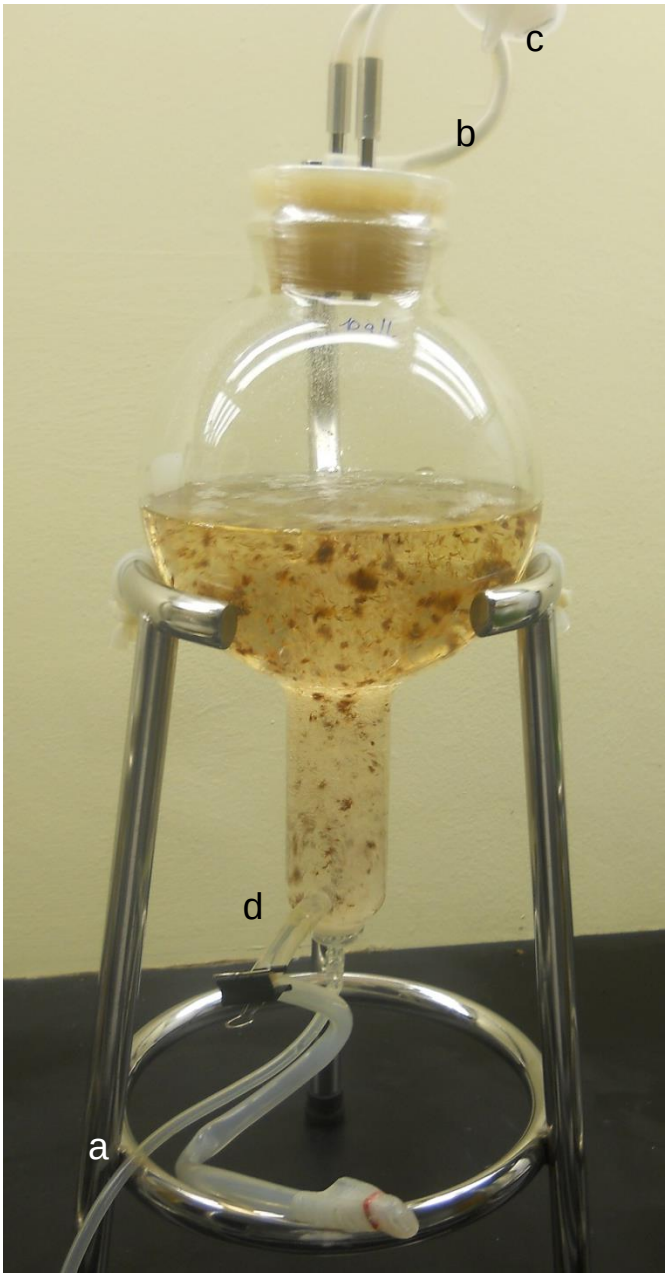

Figure 11: Design of a 3 L air-lift bioreactor after inoculation

a: open air line part connected with air pump; b: open cotton filter; c: closed air filter IN; d: closed medium line part

2.6.6. Pilot-scale bioreactor

When enough plant material was obtained in the air-lift bioreactors, the adventitious roots were transferred to the pilot-scale bioreactor. This system was used for a commercial scale production of adventitious roots of *Panax ginseng*. In Malaysian Nuclear Agency the systems contains a sterile water tank of 1000 L, a medium mixer of 1000 L, a medium sterilizer of 1000 L, an inoculation (mobile) tank of 250 L and two bioreactor vessels of 1000 L. In one of the bioreactor vessels a blade is installed to cut the roots and subculture them in an eventually bioreactor vessel of 10000 L.

Preparing the bioreactor system

The first steps that need to be done is the sterilization of all tanks and pipe lines. All of them are sterilized with steam for 1 hour at 121°C at 1,2 bar.

The next step is making the medium in the medium mixer and transferring it under high pressure to the medium sterilizer (45 minutes at 121°C at 1,2 bar). Already after the sterilization the medium can be transferred to the bioreactor vessel. This can advance the cooling of the medium.

Inoculation

Preparation of the explants:

- Choose adventitious roots from a 20 L bioreactor culture of four weeks old.
- The optimal inoculation density for a 1000 L culture is usual 5 kg of fresh adventitious roots. Around four 20 L bioreactors are enough.
- Close the air line.
- Close the cotton filter and open the air filter.
- Open the medium line of the bioreactor and wait till all the medium is gone.
- Close medium line
- Place the bioreactor in the laminar flow.

Inoculation in the mobile tank (250 L):

- Connect the air pipe line with the mobile thank and make high pressure.
- Spray the outside of the mobile tank with 70% ethanol and place it with the inoculation ort in the laminar flow.
- Flame the inoculation port and surface.
- Open the inoculation port.
- Flame again.
- Open the 20 L bioreactor.
- Use a sterile forcep to bring the adventitious roots of the four 20 L bioreactors into the mobile tank.
- Flame the inoculation port and close it.

- Gradually increase the rotational shaft speed of the blade motor to cut the inoculated roots in 1 cm long segments (700 rpm for 45 seconds).

Sterilization of the transfer pipe:

- Connect the mobile tank to the 1000 L bioreactor vessel with the transfer pipe.
- Sterilize the transfer pipe with steam (30 minutes at 121 °C).
- Wait till the pipe becomes cool.

Transfer to bioreactor vessel of 1000 L:

- Bring first 200 L medium from the bioreactor vessel into the mobile thank.
- Let it flow back under air pressure.
- Repeat this step 3 times.
- The inoculation is complete.

Elicitation

To bring the MeJA solution into the bioreactor vessel, the mobile tank is used. The method is the same as mentioned in steps above.

Harvest

Let the roots flow out under high pressure and clean them with sink water. The prevent fungi, the roots needed to be tired apart. The last part is drying them in the oven.

(Paek, 2011)

2.6.7. RITA® bioreactor

For the setup of RITA®, the liquid medium was sterilized in a Duran bottle and poured in the autoclaved (15 minutes, 121°C) RITA® in the laminar flow. After pouring the medium, the explants were placed in the culture vessel. To prevent contamination coming from the air, one-way membrane filters were mounted at the air input and output. The design of the RITA® is illustrated in figure 4.

2.6.8. SETIS™ bioreactor

To set up the SETIS™ the liquid medium was sterilized already inside the medium vessel. After autoclaving the culture and medium vessel, they were placed in the laminar flow to cool down. The explants were placed in the culture vessel when the medium was cooled down. The silicon tubes were connected at the correct place. One silicone tube connected the medium vessel with the culture vessel. The other two were used to make a connection between the medium vessel and the air compressor and between the culture vessel and the air compressor. In between a membrane filter was connected. The time cycle depends on the plant species or the sort of explant. The design of the SETIS™ is illustrated in figure 5.

3. Experimental part

3.1. Flowering of initiated and micropropagated shoots

3.1.1. Introduction

Initiation and subsequent micropropagation of the shoots is required to obtain a stock for experiments with root and leaf explants. During these phases a remarkable observation was made.

3.1.2. Specific materials and methods

The specific materials and methods were described in point 2.3, 2.4 and 2.6.1. MS medium supplemented with 0,45 mg/L BA (initiation) and 1,21 mg/L mTR (propagation) was used. The plants were placed in the culture room with a temperature of 22±2 °C and 16 h photoperiod.

3.1.3. Results and discussion

The initiated nodal segments grew out as good shoots for further use. A remarkable observation was the flowering of the initiated and propagated shoots, as shown in figure 12 and 13. After 3 weeks the flower buds were visible and later pink flowers appeared. The addition of cytokinin BA or mTR made no difference. Figure 14 shows clearly that roots were developing as well on the initiated nodal segments.

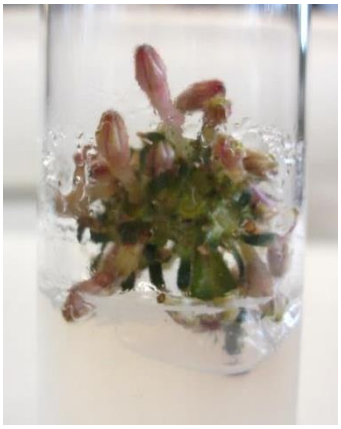

Figure 12: Flower buds on initiated shoot at 3 weeks

Figure 13: Flowers on initiated shoot at 6 weeks

Figure 14: Roots on initiated shoot at 6 weeks

3.1.4. Conclusion

MS medium supplemented with BA or mTR was suitable as initiation and stock medium. *Pentas lanceolata* flowers continuously *in vitro*. Murthy *et al*. (2012) describes that a multiplicity of factors regulate the *in vitro* flowering process (Murthy *et al.*, 2012). But it seems to be clear that *Pentas lanceolata* flowers exceptionally well *in vitro* without using specific treatment for flowering. Rooting causes no problem either.

3.2. Adventitious root induction from leaf explants on semi-solid medium with high auxin concentrations

3.2.1. Introduction

Before experiments with isolated root cultures can be performed in bioreactors, the *in vitro* roots first have to be induced. To date, no good optimized methods have yet been published regarding the induction of adventitious roots from *Pentas lanceolata*. Auxins are phytohormones used for root induction. The addition of specific exogenous auxins can trigger the differentiation and the induction pathways in rooting. It had been reported that different auxins have variable effects on different species. For instance, NAA is more potent for the induction of adventitious roots from leaf explants of *Eurycoma longiflora,* while IBA and IAA are superior for respectively *Psoralea coryfolia* and *Helianthus annuus*. (Hussein *et al.*, 2012).

In preliminary research with leaves of *Pentas lanceolata,* 4 mg/L and 8 mg/L of IBA and NAA was tested. It had been concluded that NAA was not suitable for the induction of roots. IBA seemed to be a good root inducer for *Pentas lanceolata* (Distave, 1998). In this experiment the effect of different concentrations IBA and IAA on adventitious root induction from leaf explants were examined. To see a clearly different effect between high and low concentration of auxins, a wide range of concentrations was chosen.

Also the effects of darkness and light on the root induction were compared. So the purpose of this experiment was to optimize the method for the root induction of *Pentas lanceolata* regarding light conditions and phytohormones.

3.2.2. Specific materials and methods

Medium

The semi-solid basic ½ nitrate MS medium was supplemented with 0, 2 , 4 , 6, 8 or 10 mg/L IAA or IBA and sterilized in a Duran bottle. When the medium was still liquid, it was poured in the petri dishes (described in point 2.6.2.).

Inoculation explant

For these experiments the leaves from *in vitro* shoots were cut in approx. 1 cm x 1 cm on sterile paper in the laminar flow. When the leaves were big enough, a small piece of the border was cut off to increase the occurrence of adventitious root growth. On every petri dish, 4 leaf explants were placed upside down on the medium. For each auxin concentration, 3 petri dishes were placed in darkness and 3 in light. The petri dishes were placed in the culture room with a temperature of 22±2 °C and 16 h photoperiod. For the experiment in darkness the petri dished were covered with black paper. The combination auxin free and darkness was repeated because of remarkable results.

Assessment

Four weeks long, every week the adventitious root induction was observed. Especially the start of root formation, the number of roots and the root length were noted. The data of each explant were analysed by SPSS. The analyse of variance was performed by one way ANOVA and the significant differences between the means were determined with Duncan's Multiple Range Test.

3.2.3. Results and discussion

Results IBA

Already after 1 week the callus appeared, first on the main nerves and wounds, later also on the other parts of the leaf explant. The higher the concentration of IBA the more callus was formed. Later the adventitious roots appeared out of this callus. When no IBA was added, there was no callus growing, even not after 4 weeks. Table 5 gives an overview of the observations of the different IBA concentrations after 4 weeks. This table makes clear that the induction of the adventitious roots started faster when the explants are placed in the light. Already after 2 weeks the first roots appeared. When placed in the darkness, it took 1 weeks longer. Also more adventitious roots were induced under light. Figure 16 makes a comparison between darkness and light. In the darkness a lot more callus was growing. So *Pentas lanceolata* contrasts with *Panax ginseng*, *Eurycoma longifolia and Panax notoginseng,* where the adventitious root culture is done in darkness (Kim *et al*., 2008)(Hussein *et al*., 2012) (Gao *et al*., 2005). Some other plant species prefer light, like *Labisia pumila* (Ling *et al*., 2013).

The Duncan test makes clear that the concentration 2 mg/L IBA placed in the light, with a mean of 6,5 newly formed adventitious roots, gave a significantly better result compared to the other treatments placed in the light (Figure 17). Also the mean length of the newly formed roots on 2 mg/L was higher compared to the other treatments. These results are not the same as the ones of Distave (1998). Distave showed that 8 mg/L IBA gave more roots compared to 4 mg/L IBA. The reason of this different conclusion is maybe because in this study more concentrations were tested, which gives a better overview.

The treatment without IBA was one exception on the statement that light was more suitable. None of the 12 leaf explants placed in the light formed adventitious roots, while the 12 leaf explants placed in darkness formed together a mean of 2,85 roots with a mean length of 1 cm. After 7 weeks more and longer branched roots were visible (Figure 18). The Duncan test shows that this treatment gave a significantly better result compared to the other treatments placed in the darkness. The combination of darkness and no IBA is retested several times. Still the same results were visible. This observation can possibly be explained because this is the natural growth condition of the roots (darkness under the ground without addition of exogenous auxins). It was not the optimal treatment, because roots took more time to appeare, in addition to the lower number of new roots. In general, there is a decrease of root

induction when the concentration is higher than 2 mg/L. This could be due to high concentration of auxins induces a higher level of degrader metabolites in tissue, so they block the regeneration process (Hussein *et al*., 2012).

Table 5: Effect of different IBA concentration on adventitious root induction from leaf explants after 4 weeks of culture

IBA Concentration (mg/L)	Mean adventitious roots	Mean length (cm)	Induction after … weeks
Light			
0	0,00 a		
2	6,50 b	0,35	2
4	2,08 a	0,32	3
6	0,00 a		
8	0,25 a	0,20	3
10	0,42 a	0,25	4
Darkness			
0	2,58 c	1,00	4
2	0,25 ab	0,20	3
4	0,00 a		
6	1,67 bc	0,20	4
8	0,50 ab	0,20	4
10	0,00 a		

Means within the column having the same letter are not significantly different.
Significance p<0,05, using Duncan Multiple Range Test. Post hoc tests were not performed for mean length because at least one group has fewer than two cases.

In figure 15 the mean of newly formed adventitious roots are presented in function of an increasing IBA concentration. This graph strongly indicates that over all the treatments 2 mg/L IBA is more suitable for the induction of adventitious roots.

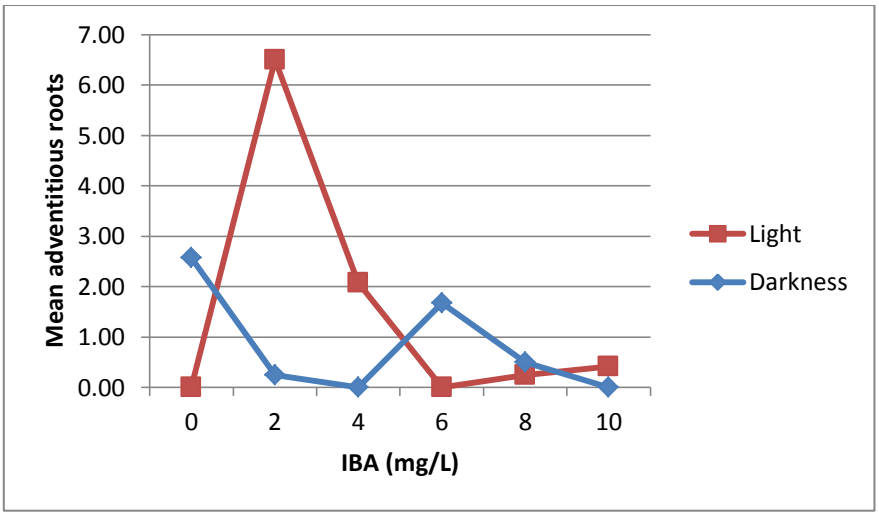

Figure 15: Effect of IBA concentration on mean adventitious roots per leaf explant in light and darkness after 4 weeks

Figure 16: Comparison between leaf explant on 4 mg/L IBA in darkness (A) and in light (B) (5 weeks)

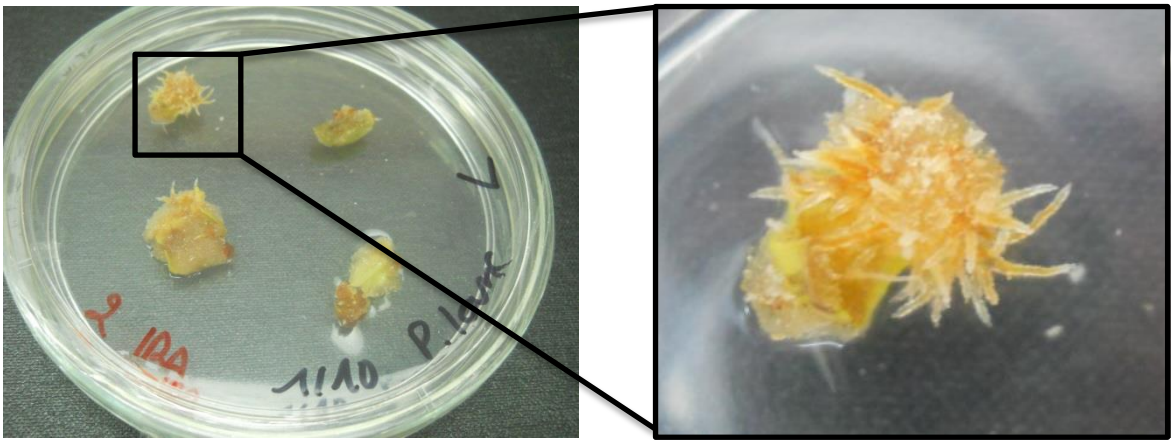

Figure 17: Leaf explants placed on 2 mg/L IBA in light (4 weeks)

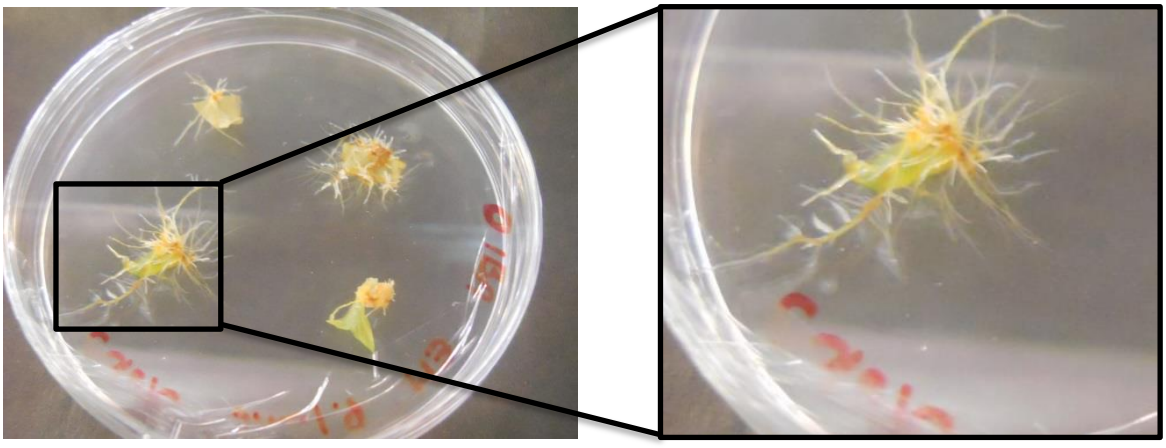

Figure 18: Leaf explants placed on medium without auxins in darkness (7 weeks)

Results IAA

The callus growth started again after 1 week, first on the nerves and cutting edges. But in general the amount of callus was lower compared to the IBA treatments. Table 6 gives an overview of the results after 4 weeks. Again it can be concluded that on the leaf explants, placed in the light, the induction of adventitious roots started faster. Already after 2 weeks the roots appeared. Here it is also more clear that the root induction in the darkness was almost negligible, except for the control treatment without IAA.

The Duncan test makes clear that the concentration 2 mg/L IAA placed in the light, with a mean of 24,25 newly formed adventitious roots, gave a significantly better results compared to the other treatments placed in the light (Figure 19). Also the mean length (0,98 cm) of the newly formed roots on 2 mg/L was significantly higher compared to the other treatments.

Table 6: Effect of different IAA concentration on adventitious root induction from leaf explants after 4 weeks of culture

IAA Concentration (mg/L)	Mean adventitious roots	Mean length (cm)	Induction after … weeks
Light			
0	0,00 a		
2	24,25 c	0,98 b	2
4	0,00 a		
6	6,42 ab	0,41 a	3
8	1,58 a	0,20 a	4
10	10,83 b	0,32 a	3
Darkness			
0	2,58 b	1,00	4
2	0,00 a		
4	0,00 a		
6	0,00 a		
8	0,00 a		
10	0,42 a	0,30	4

Means within the column having the same letter are not significantly different.
Significance p<0,05, using Duncan Multiple Range Test. Post hoc tests were not performed for mean length in darkness because there are fewer than three groups.

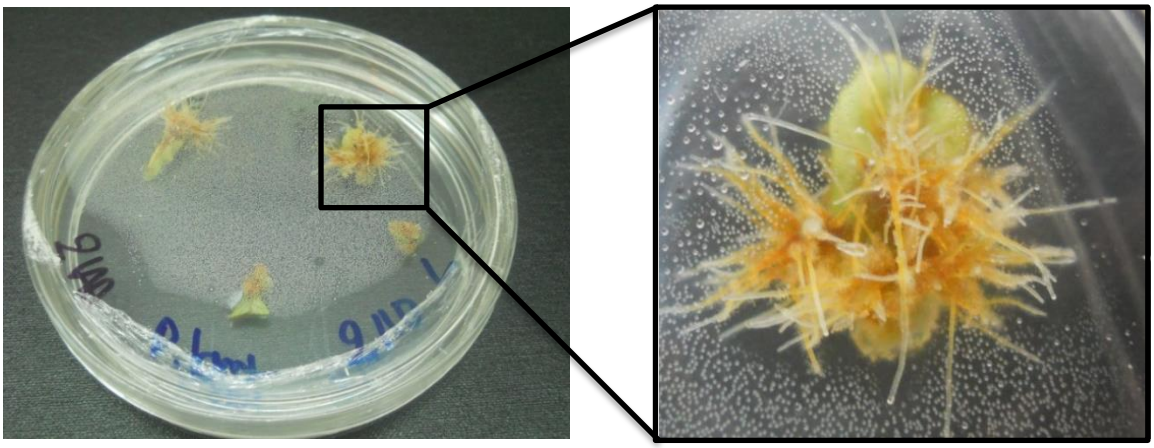

Figure 19: Leaf explants placed on 2 mg/L IAA in light (4 weeks)

In figure 20 the mean of newly formed adventitious roots are presented in function of an increasing IAA concentration. This graph strongly indicates that over all the treatments 2 mg/L IAA is more suitable for the induction of adventitious roots.

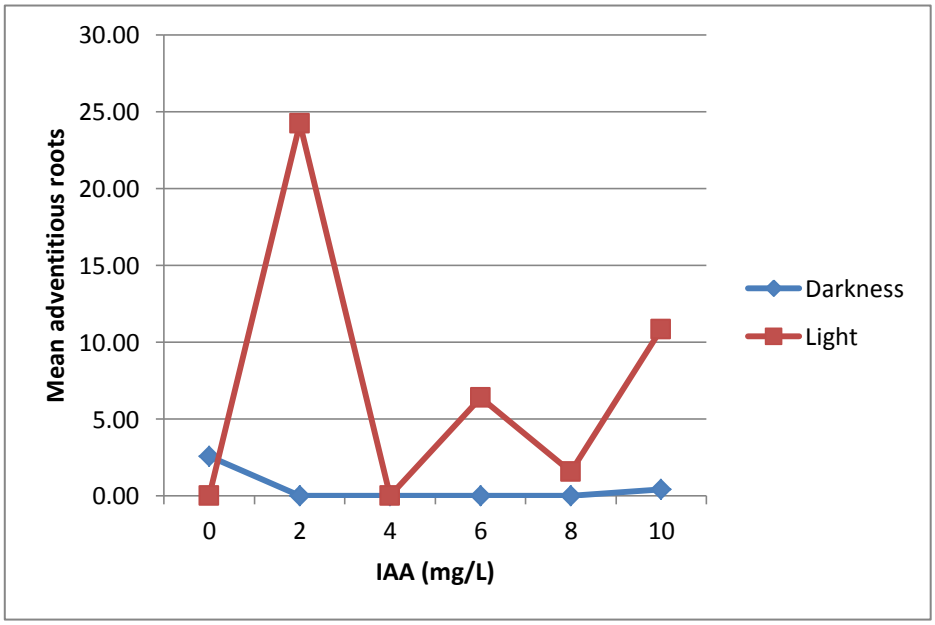

Figure 20: Effect of IAA concentration on mean adventitious roots per leaf explant in light and darkness after 4 weeks

3.2.4. Conclusion

If the results of the two auxins IBA and IAA are compared, it can be concluded that in both auxin treatments the induction of adventitious roots is the best when the explants are placed in the light. There is one remarkable exception. When there is no exogenous auxin added, the mean number of adventitious roots is 2,58 in the darkness. But this amount is still low if compared with the other treatments and it takes a longer time to induce the roots.

The best treatment found for the auxin IBA was 2 mg/L with a mean of 6,5 adventitious roots after 4 weeks. Out of the IAA treatments 2 mg/L IAA gave the best result with 24,25 adventitious roots after 4 weeks.

Thus out of these experiment can be concluded that, of all tested concentrations, the treatment with 2 mg/L IAA placed in the light was the most suitable condition for the induction of adventitious roots from leaf explants. Of course lower concentration should be tested too. That is why next experiment was set up.

3.3. Adventitious root induction from leaf explants on semi-solid medium with low auxin concentrations

3.3.1. Introduction

Experiment 3.2. showed that lower auxin concentration induce more adventitious roots. Therefore the lower concentrations 1, 2 and 3 mg/L IAA and 1, 1,5, 2 and 3 mg/L IBA were examined in this experiment. Because experiment 3.2 showed clearly that in the light more roots are induced, this experiment was only conducted in the light.

3.3.2. Specific materials and methods

Medium

The semi-solid basic ½ nitrate MS medium was supplemented 1, 2 and 3 mg/L IAA and 1, 1,5, 2 and 3 mg/L IBA and sterilized in a Duran bottle. When the medium was still liquid, it was poured in the petri dishes (described in point 2.6.2).

Inoculation explant

The leaves from *in vitro* shoots were cut in approx. 1 cm x 1 cm on sterile paper in the laminar flow. For each treatment 2 petri dishes were used and each petri dish was inoculated with 6 leaf explants. The petri dishes were placed in the culture room with a temperature of 22±2 °C and 16 h photoperiod.

Assessment

No statistical analysis has been done. After 3 and 6 weeks development was compared visually.

3.3.3. Results and discussion

The appearance of the adventitious roots from the leaf explants looked to be the same in every treatment. Green parts of the leaves were still visible. The roots grew out of dark coloured callus that was growing on the nerves and cutting edges. Usually more roots grew out of the callus on the leafstalk side. The base of the roots was orange/brown, the youngest parts were lighter. The older roots were darker because they also developed callus. The roots were long and branched. The lateral roots seemed to grow out of callus spheres.

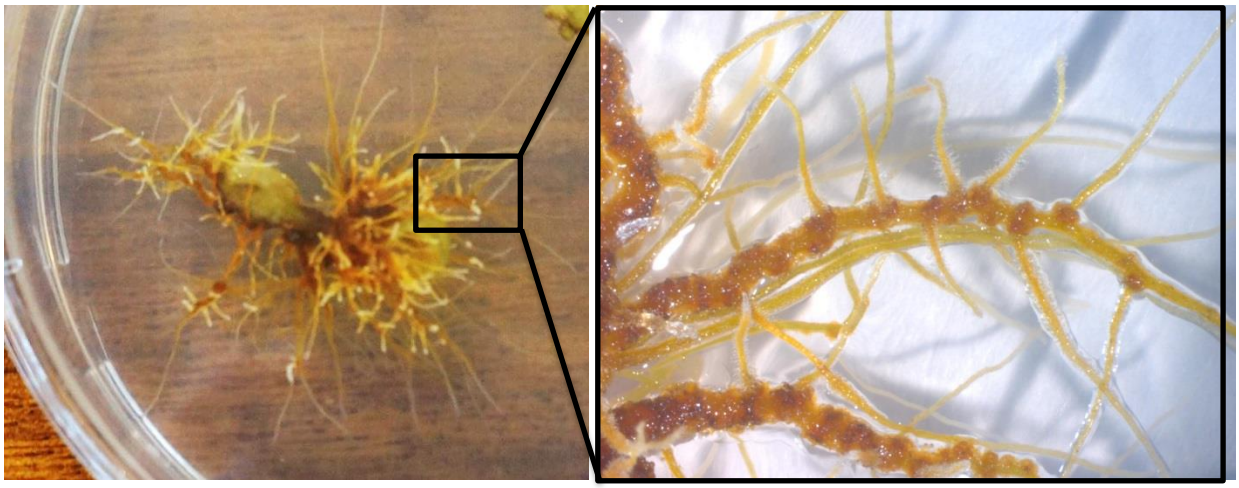

Figure 21: Leaf explant on 1 mg/L IBA in light (6 weeks)

Results IBA

After three weeks in culture most adventitious roots were visible in the treatments 1 mg/L and 3 mg/L. Three weeks later, the number of roots in the treatment 1 mg/L was obviously higher and the roots were more branched. The explants on the medium with 1,5 mg/L had more callus production, the treatments with 2 and 3 mg/L even more.

Results IAA

These results showed almost the same pattern. After three weeks on 1 mg/L IAA, two times more adventitious roots were induced in comparison with 1 mg/L IBA. The concentrations 2 and 3 mg/L induced less roots. After 6 weeks in culture, still more roots were growing on 1 mg/L. Higher concentrations showed more callus and shorter roots. When the auxin concentration increased, more callus was growing and the number of induced roots decreased.

3.3.4. Conclusion

Again can be concluded that IAA induces more adventitious roots. The concentration of 1 mg/L gives in both cases the best result. When the concentration increases, the number and length of roots decreases. Low auxin concentrations might be better due to the lower production of callus. In medium with higher auxin concentrations the roots have to compete with the fast growing callus.

3.4. Root induction from root explants in semi-solid medium with high auxin concentrations

3.4.1. Introduction

Due to the fact that *Pentas lanceolata* roots easily *in vitro* (Figure 14), roots can also be isolated from the shoot cultures and used for root induction experiments. To date, no optimized methods have yet been published regarding the induction of roots out of root explants from *Pentas lanceolata.*

In this experiment the effect of different auxins IBA, IAA and NAA were examined each in darkness and light. As in the experiment with the leaf explants a wide range of concentrations was chosen. The purpose of this experiment was to find the best conditions (phytohormones and light conditions) to induce new roots out of root explants.

3.4.2. Specific materials and methods

Medium

The semi-solid basic ½ nitrate MS medium was supplemented with 0, 2 , 4 , 6, 8 or 10 mg/L IAA, IBA or NAA and sterilized in a Duran bottle. When the medium was still liquid, it was poured in the petri dishes (see 2.6.2.).

Inoculation explant

Roots of *in vitro* shoots were cut in pieces of approx. 1-2 cm and initiated on the semi-solid medium. For every auxin concentration 3 petri dishes were placed in darkness and 3 in light. Every petri dish contains 9 root explants. The petri dishes were placed in the culture room with a temperature of 22±2 °C and 16 h photoperiod. For the experiment in darkness the petri dished were covered with black paper. Because the combination auxin free medium placed in darkness gave remarkable results, these were tested twice. The same happened for the combination 10 mg/L IAA placed in light.

Assessment

Four weeks long, every week the root induction was observed. Especially the start of root formation, the number new roots and the length of new roots were noted. The data of each explant were analysed by SPSS statistical analysis. The analyse of variance was performed by one way ANOVA and the significant differences between the means were determined Duncan's multiple range test.

3.4.3. Results and discussion

Results IBA

After 1 week callus was already visible. First on parts of the explant, later over the whole root. Later, the new roots were developing from this callus. The higher the concentration the more callus was growing. The treatment without addition of exogenous auxins gave again a remarkable result. In light and darkness there was hardly no callus growing, even not after 4 weeks.

Table 7 gives an overview of the observations of the different IBA concentrations after 4 weeks. This table makes clear that the induction of the new roots started faster when the explants were placed in the light. Already after 2 weeks the first roots appeared. Also more new roots were induced in the light. Figure 22 makes a comparison between darkness and light. In the darkness a lot more callus was produced.

The Duncan test makes clear that the concentration 2 mg/L IBA placed in the light, with a mean of 8,07 newly formed new roots, gave a significantly better result compared to the other treatments placed in the light (Figure 23). Also the mean length (0,45 cm) of the newly formed roots on 2 mg/L was significantly higher compared to the other treatments.

There was one exception on the statement that light was more suitable. None of the 24 roots explants placed in the light on auxin free medium formed new roots, while explants on auxin free medium placed in the darkness had a mean of 0,93 newly formed roots with a mean length of 1,2 cm (Figure 24). Already after 1 week the first roots appeared. The Duncan test shows that this treatment gave a significantly different result compared to the other treatments placed in the darkness. The combination of darkness and no IBA was retested several times, which gave comparable results. This observation can possibly be explained because it is the natural growth condition of the roots (darkness under the ground without addition of exogenous auxins).

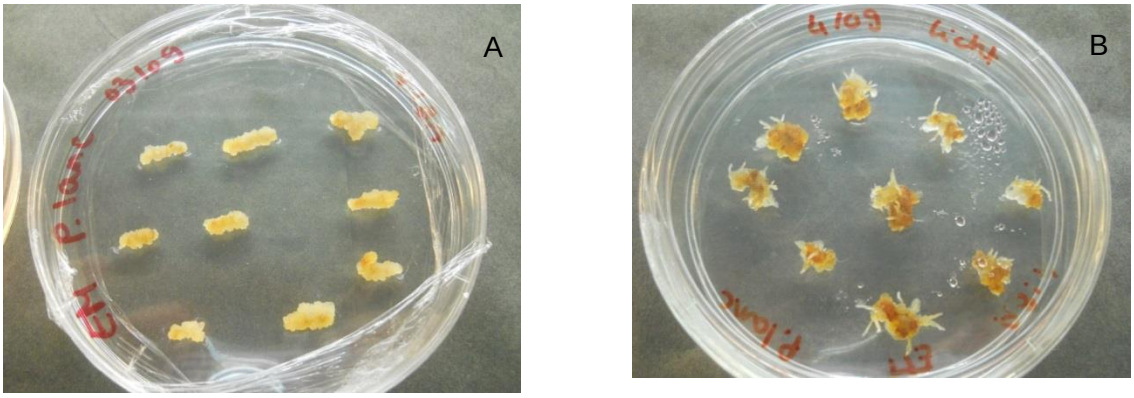

Figure 22: Comparison between root explants on 4 mg/L IBA in darkness (A) and in light (B) (3 weeks)

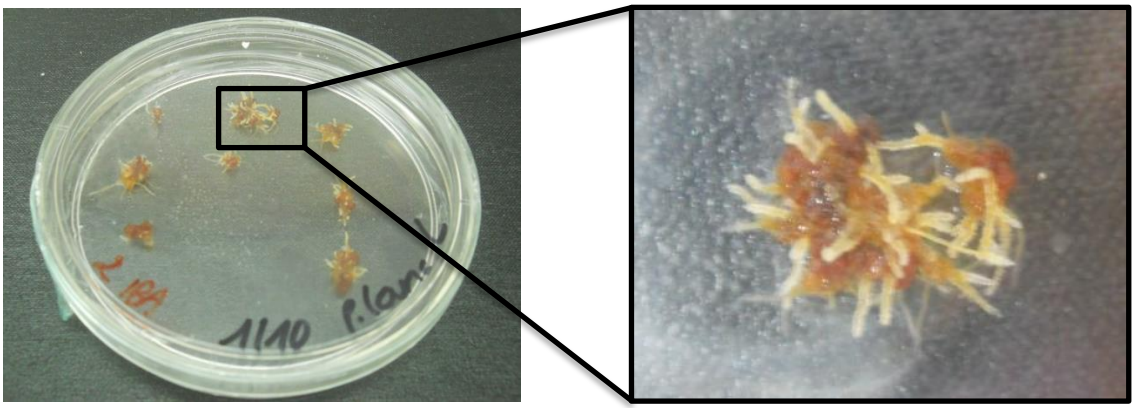

Figure 23: Root explants on 2 mg/L IBA in light (4 weeks)

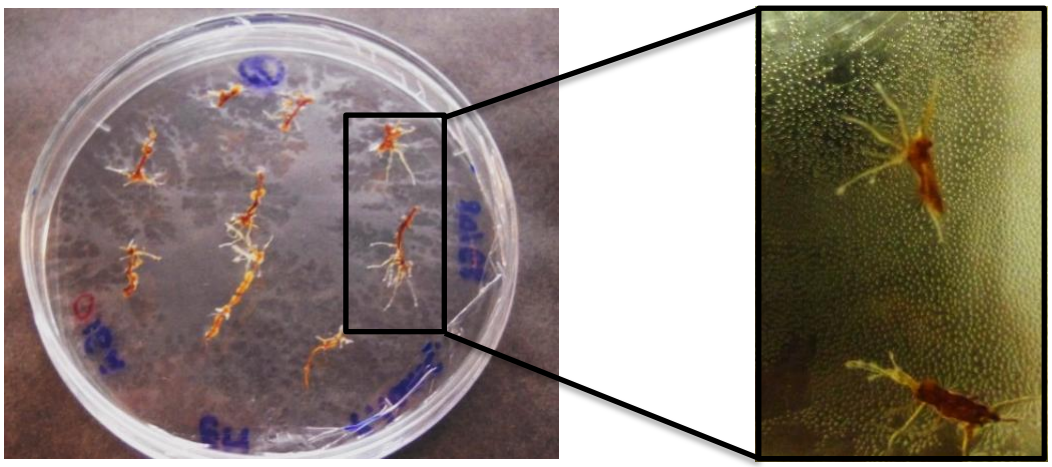

Figure 24: Root explants placed on medium without auxin in darkness (4 weeks)

Table 7: Effect of different IBA concentration on root induction on root explants after 4 weeks of culture

IBA Concentration (mg/L)	Mean new roots	Mean length (cm)	Induction after … weeks
Light			
0	0,00 a		
2	8,07 c	0,45 b	2
4	2,63 b	0,30 a	2
6	1,15 ab	0,28 a	2
8	2,89 b	0,25 a	2
10	0,00 a		
Darkness			
0	0,93 b	0,93	1
2	0,07 a	0,20	3
4	0,00 a		2
6	0,00 a		
8	0,00 a		
10	0,00 a		4

Means within the column having the same letter are not significantly different.
Significance p<0,05, using Duncan Multiple Range Test. Post hoc tests were not performed for mean length in darkness because there are fewer than three groups

Figure 25 makes a comparison between the mean new roots after 4 weeks in light and darkness. This graph strongly indicates that when placed in the light, more new roots are formed and that concentration 2 mg/L IBA gives the best result.

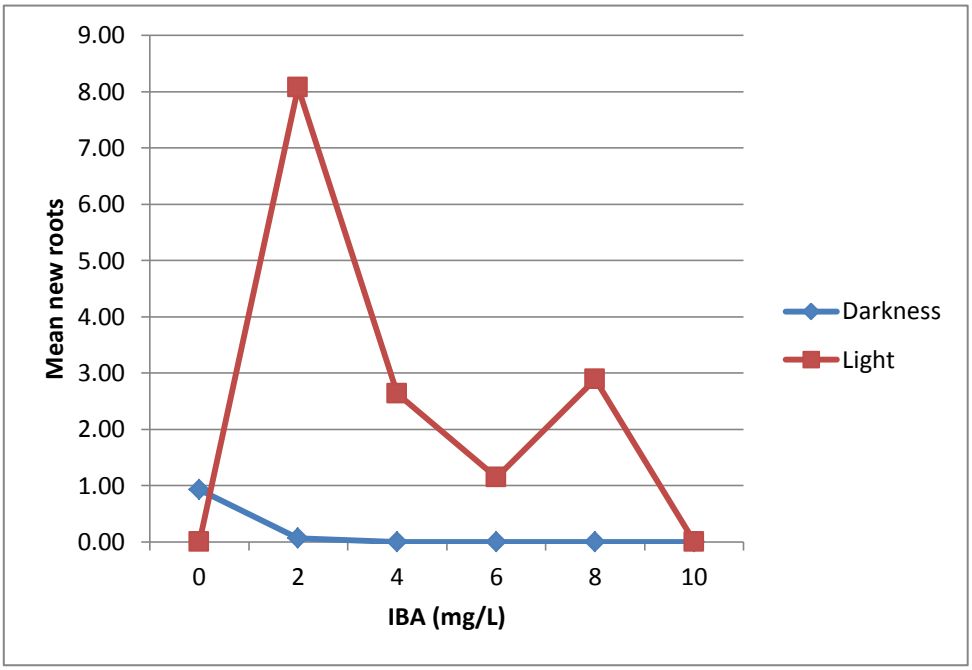

Figure 25: Effect of IBA concentration on mean new roots per root explant in light and darkness after 4 weeks

Results IAA

Table 8 gives an overview of the observations of the different concentrations after 4 weeks. This table makes clear that in all the treatments roots appeared after 2 weeks. In general, most roots were induced when placed in the light.

According to the Duncan test, roots on 10 mg/L IAA placed in the light gave the best result, with a mean of 13,85 newly formed roots per root explant (Figure 26). This concentration also yielded the highest mean root length (0,98 cm). The concentration 2 and 6 mg/L IAA induced also roots. The mean number of roots, respectively 3,93 and 3,41, were rather low compared to 10 mg/L. This is not an expected result. Usually more callus grows at a high concentration of auxins. This treatment was retested. Again the same results were visible. Perhaps a high concentration of IAA suppresses the auxin which gives the roots more chance to grow.

The concentration 4 mg/L IAA gave no new roots after 4 weeks. Maybe something went wrong with the preparation of the medium or the roots where dried out before transferring them to the petri dish.

Figure 26: Root explants placed on medium with 10 mg/L IAA in light (4 weeks)

Table 8: Effect of different IAA concentration on root induction on root explants after 4 weeks of culture

IAA Concentration (mg/L)	Mean new roots	Mean length (cm)	Induction after … weeks
Light			
0	0,00 a		
2	3,93 b	0,31 a	2
4	0,00 a		
6	3,41 ab	0,24 a	2
8	1,30 ab	0,26 a	2
10	13,85 c	0,54 b	2
Darkness			
0	0,93 c	0,93 b	1
2	0,48 b	0,34 a	2
4	0,00 a		
6	0,30 ab	0,23 a	2
8	0,00 a		
10	0,00 a		

Means within the column having the same letter are not significantly different.
Significance p<0,05, using Duncan Multiple Range Test.

Figure 27 makes a comparison between the mean new roots after 4 weeks in light and darkness. This graph strongly indicates that when placed in the light, more new roots are formed and that concentration 10 mg/L IAA gives the best result.

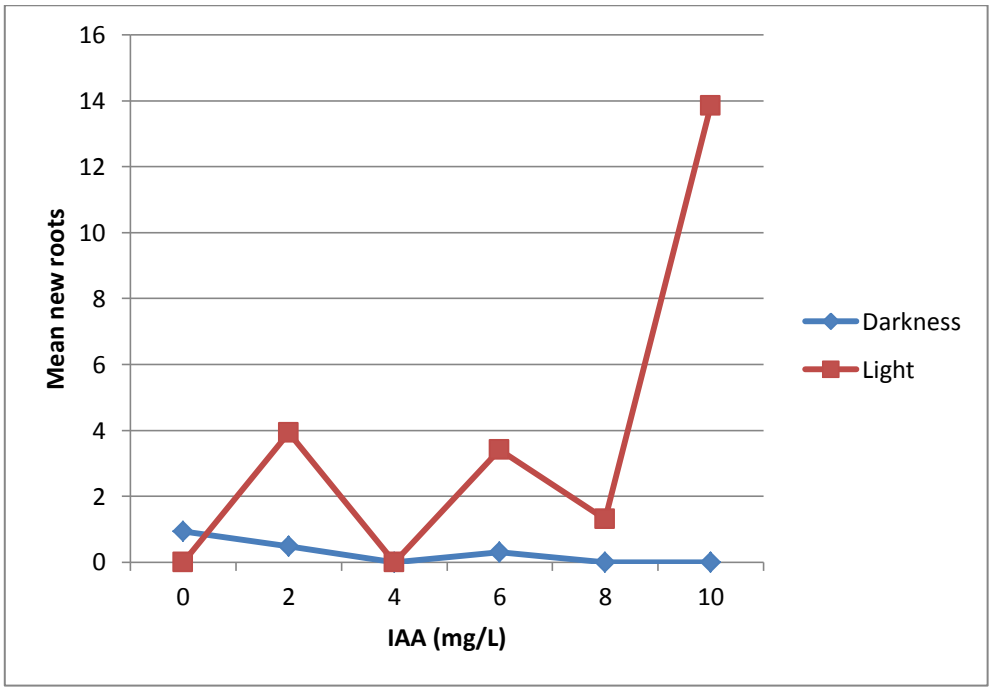

Figure 27: Effect of IAA concentration on mean new roots per root explant in light and darkness after 4 weeks

Results NAA

When the treatments with NAA were observed, it was remarkable that the root induction started faster (after 1 week) and the callus production was lower compared to the treatments with IAA and IBA. When the concentration increased the callus production decreased. This is different from the other auxins.

The Duncan test makes clear that the concentration 10 mg/L NAA, 6 mg/L and 2 mg/L placed in the light gave a significantly different result compared to the other treatments placed in the light (Figure 9). As in the treatment with IBA, the concentration with the highest number of new roots had also the highest mean root lenght. The mean length (0,37 cm) of the newly formed roots on 10 mg/L was higher compared to the other treatments. The concentration 2 and 6 mg/L IAA induced also roots. The means, respectively 1,78 and 0,96, were not significantly different compared to 10 mg/L with a mean of 2,04.

The explants with concentration 10 mg/L NAA in the darkness gave almost the same amount of roots. The mean was here 1,89 newly formed roots. Notable was the high mean length of 0,77 cm. But in general, the amount of new roots with NAA was much lower than with the other auxin treatments.

Table 9: Effect of different NAA concentration on root induction on root explants after 4 weeks of culture

NAA Concentration (mg/L)	Mean new roots	Mean length (cm)	Induction after … weeks
Light			
0	0,00 a		
2	1,78 bc	0,24	1
4	0,44 ab	0,25	2
6	0,96 abc	0,27	3
8	0,07 a	0,20	4
10	2,04 c	0,37	1
Darkness			
0	0,93 b	0,93	1
2	0,78 b	0,26	2
4	0,07 a	0,20	3
6	0,00 a		
8	0,00 a		
10	1,89 c	0,77	1

Means within the column having the same letter are not significantly different.
Significance p<0,05, using Duncan Multiple Range Test. Post hoc tests are not performed for mean length because at least one group has fewer than two cases.

Figure 28 makes a comparison between the mean new roots after 4 weeks in light and darkness. This graph indicates that a high concentration causes more adventitious roots in light and in darkness. But the mean is still very low.

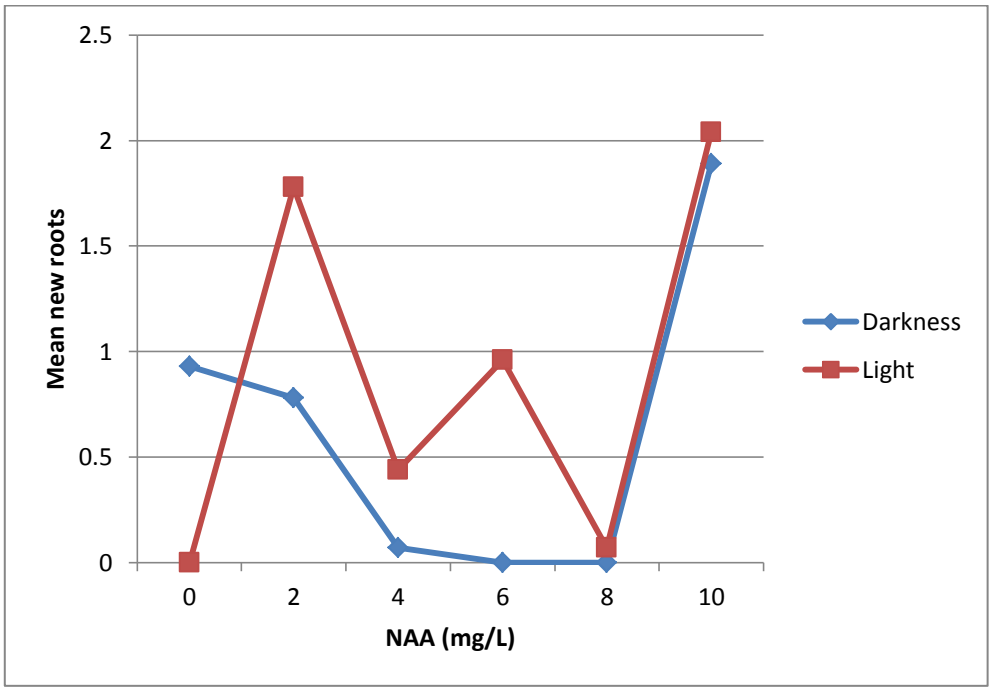

Figure 28: Effect of NAA concentration on mean new roots per root explant in light and darkness after 4 weeks

3.4.4. Conclusion

Generally, for each auxin, the results of the root explants placed in the light are much better than the ones placed in the darkness. This is also remarkable in the experiments with the leaf explants, just like the treatment without auxins placed in the darkness. But in auxin free medium, new roots develop on the root explants after already 1 week in contrast to 4 weeks on the leaf explants. After 4 weeks the mean amount of the newly formed roots on the root explants is only 0,93 roots. Leaf explant develop more roots (2,5 per leaf).

With a mean of 8,07 of newly formed roots on root explants after 4 weeks and a mean length of 0,45 cm, 2 mg/L IBA is the best concentration out of all the IBA treatments. It can also be concluded that root explants react better on IBA than the leaf explants.

The best concentration of IAA is 10 mg/L with a mean of newly formed roots of 13,85 on leaf explants after 4 weeks. It was clear that the leaf explants (mean of 24,25 roots) react better on IAA than the root explants.

The trigger of NAA on the root explants is very low. The highest mean of newly formed roots is 2,04 roots in the treatment with 10 mg/L NAA. Maybe higher doses of NAA need to be tested.

3.5. Root induction from root explants in semi-solid medium with low auxin concentrations

3.5.1. Introduction

Experiment 3.4. showed that on root explants lower IBA concentration induce more new roots. Therefore the lower concentrations 1, 1,5, 2 and 3 mg/L IBA are examined. The experiment with IAA showed a significant higher amount of new roots at 10 mg/L IAA. But also for IAA these concentrations 1 ,2 and 3 mg/L are tested. Because experiment 3.4. showed clearly that under the light more roots are induced, this experiment was only conducted in light.

3.5.2. Specific materials and methods

Medium

The semi-solid basic ½ nitrate MS medium was supplemented with 1, 2 and 3 mg/L IAA and 1, 1,5, 2 and 3 mg/L IBA and sterilized in a Duran bottle. When the medium was still liquid, it was poured in the petri dishes (see point 2.6.3.).

Inoculation explant

Roots originated from *in vitro* shoots were cut in pieces of approx. 1-2 cm and initiated on the semi-solid medium. For each treatment 2 petri dishes were used and each petri dish was filled with 9 root explants. The petri dishes were placed in the culture room with a temperature of 22±2 °C and 16 h photoperiod.

Assessment

No statistical analysis has been done. After 3 and 6 weeks the development was compared visually.

3.5.3. Results and discussion

The development of new roots on the root explants looked the same in every treatment. Generally, the root explant was overgrown with dark coloured callus. Out of these callus the light coloured short roots were growing. The base of the roots was darker, the youngest parts were lighter. The older parts were darker because they also developed callus (Figure 29). The induction of adventitious roots out of leaf explants resulted in more and longer roots with less callus production (Figure 21).

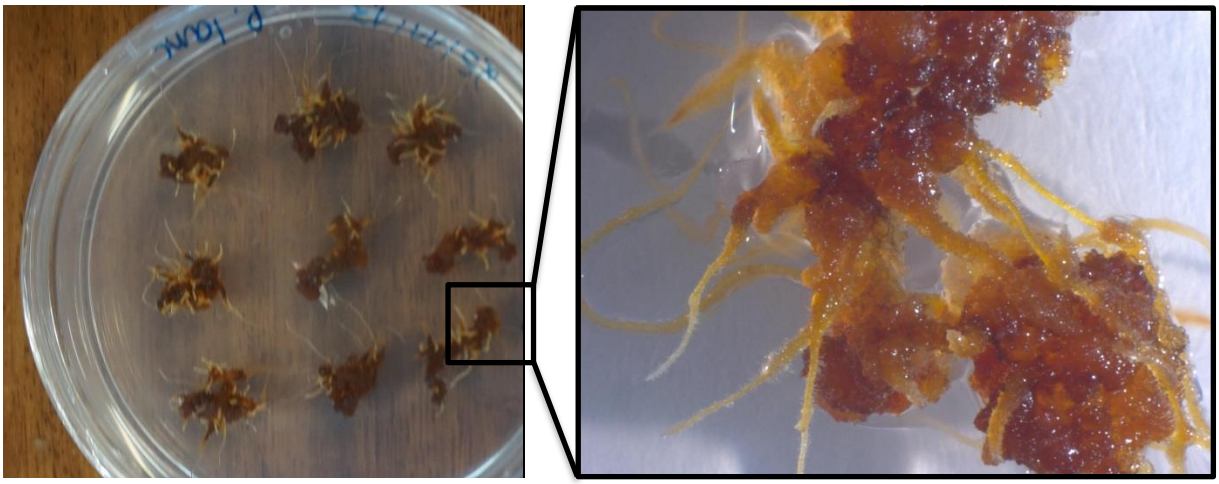

Figure 29: Root explants on 1 mg/L IBA in light (6 weeks)

Results IBA

After three weeks the treatment with 1 mg/L induced most new roots. The higher the concentration the lesser new roots were formed. Three weeks later the same pattern was visible. The number of new roots on 1 mg/L was higher than the other concentrations and less callus was formed.

Results IAA

The number of new roots formed on 1 mg/L IAA was half the number of the roots formed on 1 mg/L IBA after 3 weeks. The roots were also shorter. The higher concentrations 2 mg/L and 3 mg/L showed even lower numbers. After 6 weeks the same pattern was visible. It was remarkable that the treatment with 1 mg/L produced the least callus.

3.5.4. Conclusion

It can be concluded that IBA is more potent to induce new roots on root explants in lower concentrations. Earlier was concluded that 10 mg/L IAA was more suitable compared to 2 mg/L IBA. But 1 mg/L IBA gave a visible better result than 10 mg/L IAA. The concentration of 1 mg/L gives in both cases (IBA and IAA) the best result. When the concentration increases, the number and length of the roots decreases. Low auxin concentrations might be better due to the lower production of callus. In higher auxin concentrations the roots have to compete with the fast growing callus.

Out of these last four experiments can be concluded that the best treatment, for the induction of adventitious roots from leaf explants, is 1 mg/L IAA. Then the isolated roots have to be subcultured. On solid medium the best treatment is 1 mg/L IBA.

3.6. Acute gamma irradiation on leaf and root explants

3.6.1. Introduction

One of the most common techniques to induce plant mutations is an irradiation treatment. The mutants out of these treatment can be useful to develop new varieties or for functional gene studies. Maybe mutations with an increased production of interesting secondary metabolites or with a better root growth could be found.

This experiment was carried out to study the effect of acute gamma irradiation on the induction of adventitious roots on leaf and root explants of *Pentas lanceolata*. Out of the results the LD50 can be found. LD50 is the dose of gamma rays at which 50% of the explants will die. This is also the point where the most mutations can be created. With LD50 the effective doses for the *in vitro* mutagenesis can be found.

3.6.2. Specific materials and methods

Before acute irradiation

One day before the irradiation treatment, the root and leaf explants were placed on a basic semi-solid MS medium without hormones. For each treatment 3 petri dishes were used. The root explants were placed by 9 and the leaf explants by 4 in a petri dish.

Treatment

The explants were irradiated with acute gamma irradiation at 0, 10, 20, 50, 70, 100 Gy by using the Gamma Cell facility of the institute Malaysia Nuclear Agency. After irradiation the explants were transferred to another petri dish filled with semi-solid ½ nitrate MS medium supplemented with 4 mg/L IBA. The petri dishes were placed in the culture room with a temperature of 22±2 °C and 16 h photoperiod.

Assessment

After 4 weeks of culture, the survival rate of the explants and number and length of the new adventitious roots were measured. The data were analyzed by SPSS using one way ANOVA with Duncan's multiple range test.

3.6.3. Results and discussion

The survival rate of the leaf explants cannot be used to determine the LD50 (Table 10). The leaves still survived at the highest dose, also the number of adventitious roots gave no declining curve. The results of the root explants were more suitable to find the LD50 (Table 11). The survival rate showed a slowly declining curve. But at 100 Gy still 77,7% of the roots was alive. This is not the expected result. Usually plants die at the dose of 100 Gy (Sajahan *et al*.,

2013; Jala *et al*., 2011). The number of new roots formed on the root explant decreased more. With a dose of 100 Gy only a mean of 0,59 roots were formed. So the graph of this observations can be used to find the equivalent of LD50. Figure 30 makes clear that with an increasing gamma dose, the number of new roots declines significantly. Based on the linear trendline of the graph, the 'LD50' is located around 47 Gy. The LD100 might be around 93 Gy.

Table 10: Effect of different doses acute gamma irradiation on leaf explants after 4 weeks of culture

Dose (Gy)	Mean adventitious roots from leaf explants	Survival rate (%)	Mean length (cm)
0	0,16	100	0,40
10	0,60	100	0
20	0,00	100	0,37
50	0,58	100	0,26
70	1,83	100	0,27
100	0,00	100	0

Post hoc tests are not performed because at least one group has fewer than two cases.

Table 11: Effect of different doses acute gamma irradiation on root explants after 4 weeks of culture

Dose (Gy)	Mean new roots from root explants	Survival rate (%)	Mean length (cm)
0	4,67 a	100 a	0,30 a
10	3,00 a	92,5 ab	0,24 ab
20	3,22 a	96,2 a	0,26 ab
50	1,04 b	96,2 a	0,20 a
70	0,21 b	88,8 ab	0,23 ab
100	0,59 b	77,7 b	0,20 b

Means within the column having the same letter are not significantly different.
Significance p<0,05, using Duncan Multiple Range Test.

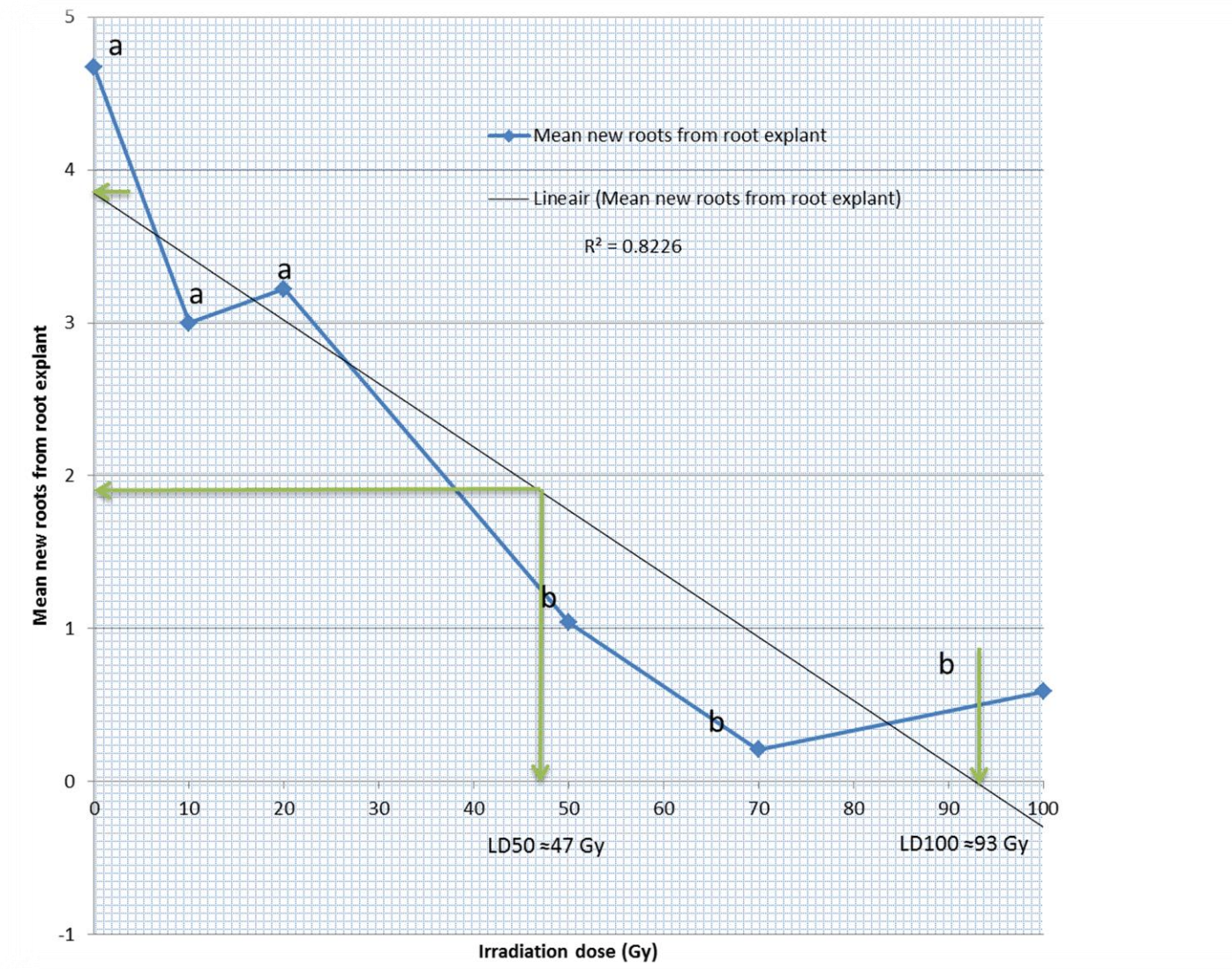

Figure 30: Mean new roots from root explants after 4 weeks in culture

Means having the same letter are not significantly different.
Significance p<0,05, using Duncan Multiple Range Test.

3.6.4. Conclusion

The dosis with 50% root induction on root explants of *Pentas lanceolata* in this experiment is located around 47 Gy. So the effective doses to become mutations should be selected at 37 Gy, 47 Gy and 57 Gy. This range around the 'LD50' normally cause the most functional mutations. There are some questions about the results of this experiment. Still a lot of explants survive at high doses. These experiment need to be repeated to be sure about these conclusion. Maybe it is also better to transfer the explants on the most suitable medium. The concentration of 4 mg/L IBA is not the most optimal concentration for root induction. At the time of the irradiation experiment this was not known yet.

3.7. Adventitious root induction from leaf explants in liquid medium

3.7.1. Introduction

To check if it is possible to induce adventitious roots on leaf explants in liquid medium, different concentrations of IBA were tested. Earlier experiments made already clear that root induction goes much better in light conditions. So the different concentrations are only tested in light.

3.7.2. Specific materials and methods

Medium

The liquid ½ nitrate MS medium was supplemented with 0, 2 , 4 ,6, 8 and 10 mg/L IBA . The method using shake flasks is described in point 2.6.3.

Inoculation explant

For each treatment one Erlenmeyer of 100 ml was filled with 50 ml medium and 6 leaf explants of around 1 cm x 1 cm. The Erlenmeyers were placed in the culture room with a temperature of 22±2 °C and 16 h photoperiod.

Assessment

No statistical analysis has been done. Every week development was compared visually.

3.7.3. Results and discussion

After 1 week the colour of the leaves changed from green to brown. Only the leaf explants that were placed in auxin free medium remained green.

The leaves in auxin free medium were still green after 2 weeks in culture. In 2 mg/L IBA some callus was growing on the nerves at the backside of the leaves. In the medium supplemented with 4 and 6 mg/L IBA, more callus was growing over the whole leaf. The leaves of concentration 8 and 10 mg/L showed a colour change from brown to white.

After 3 weeks, the leaves in auxin free medium were still green but some callus was growing on the cutting site. In 2 mg/L IBA the callus was growing over the whole leaf. At two leaves four adventitious roots with an average length of 0,3 cm were growing. The callus grown on the leaves in 4 and 6 mg/L IBA fragmented and swirled in the medium and the leaves in the higher concentrations started to produce callus on the nerves.

After 4 weeks in auxin free medium, three roots with an average length of ± 0,4 cm were growing on one leaf. But more roots were growing in the treatment with 2 mg/L IBA. On each

leaf different roots were growing with an average length of 1,2 cm. The callus on the leaves had a dark brown colour and fragments swirled around in the medium. The situation in the other treatments remained the same.

The results after 6 weeks are shown in figure 31. In the treatment without IBA fourteen roots were growing out of the cutting place. The length of the roots was ± 2 cm. The 6 leaves placed in 2 mg/L IBA produced around 30 new roots. The length of these roots was ± 3 cm.

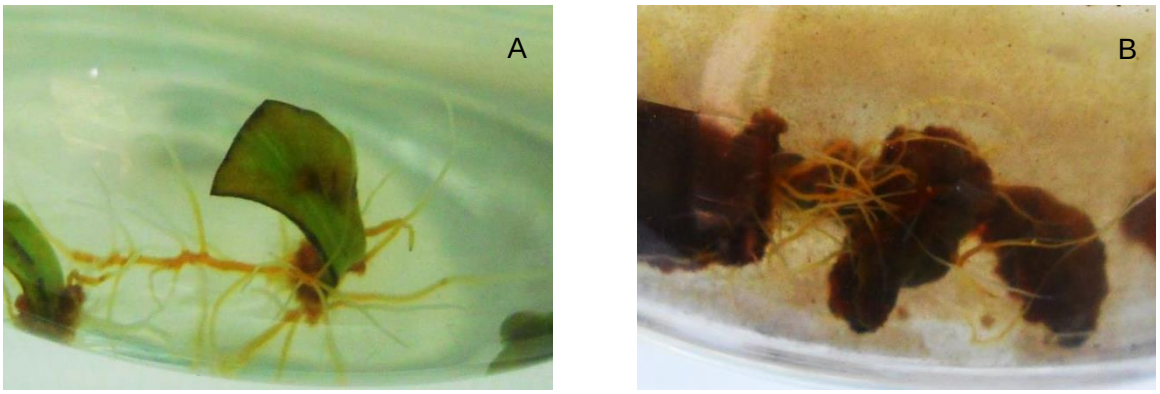

Figure 31: Leaf explants in liquid ½ nitrate MS medium without auxins (A) and supplemented with 2 mg/L IBA (B) (6 weeks)

3.7.4. Conclusion

Out of this experiment can be concluded that it is possible to induce adventitious roots out of leaf explants who are placed in liquid medium. In the medium supplemented with 2 mg/L IBA the most roots are formed. But if the results are compared with the induction of new roots out of root explants, the yield out of leaf explants is much lower and it takes a longer time (6 weeks) before a bigger amount of roots is induced.

It is possible that the tested conditions are not the most suitable. Maybe less medium need to be used and the auxin IAA should also be tested. Because earlier described experiments showed that IAA is more potent for the induction of adventitious root from leaf explants

3.8. Multiplication of adventitious roots in liquid medium

3.8.1. Introduction

To prepare the roots for the bioreactor, the isolated roots need to be transferred to an Erlenmeyer with liquid medium on a shaking table. The optimal conditions can differ from the ones in the semi-solid medium. So the light conditions and the effects of the auxins IBA and IAA need to be tested on the isolated roots. Distave (1998) tested 2 mg/L IBA and 4 mg/L IBA and concluded that 2 mg/L IBA was the most suitable concentration for the growth of adventitious roots in liquid medium. In this experiment the light condition and concentration of IBA and IAA were tested to find the best condition to multiply a lot of roots in liquid medium.

3.8.2. Specific materials and methods

The method for the use of the shake flask is described in point 2.6.3.

Light conditions

Adventitious roots from of leaf explants were used to test the light conditions. Before transferring the roots they were cut in pieces of approx. 1-2 cm. They were transferred into 10 Erlenmeyers of 100 ml with 50 ml liquid ½ nitrate MS medium supplemented with 5 mg/L IBA. The roots have to swirl in the medium (±7 g/L). Five of these Erlenmeyers were placed in darkness and five in light. The Erlenmeyers were placed in the culture room with a temperature of 22±2 °C and 16 h photoperiod. For the experiment in darkness the Erlenmeyers were placed in a box.

IBA concentration

To find the optimal IBA concentration roots formed out of root explants were used. Before transferring the roots they were cut in pieces of approx. 1-2 cm. The concentrations of 2 mg/L and 4 mg/L IBA were tested in light. Also the medium without IBA was used and placed in darkness. The Erlenmeyers were placed in the culture room with a temperature of 22±2 °C and 16 h photoperiod. For the experiment in darkness the Erlenmeyers were placed in a box.

IAA concentration

Earlier described experiments made clear that the auxin IAA is also potent to induce adventitious roots on *Pentas lanceolata* explants on semi-solid medium. It was also obvious that the roots from leaf explants contains less callus. So adventitious roots grown out of leaf explants were transferred to liquid medium supplemented with 2 mg/L IAA. The Erlenmeyers were placed in the culture room with a temperature of 22±2 °C and 16 h photoperiod.

Assessment

No statistical analysis has been done. Every week development was compared visually.

3.8.3. Results and discussion

Light conditions

- After 1 week all the roots turned darker and a thin layer of callus was visible. There was no difference between the explants placed in the darkness or in the light.
- After 2 weeks the medium became cloudy, both in light and darkness. Also more callus was produced and the first small adventitious roots appeared.
- When the explants were 3 weeks in culture the adventitious roots became more clear. The roots placed in the light had longer adventitious roots than the ones placed in the darkness.
- After a cultivation of 4 weeks the medium was turned dark brown and needed refreshment. The newly formed roots in the light were still longer and more callus was found on the roots in the darkness.

Figure 32 and 33 show the result of the new adventitious roots formed after 5 weeks. The new roots grown in darkness (Figure 32) were short and dense, the longest roots were ± 1 cm long. Also a lot of callus was visible on the root explants. Figure 33 shows the new roots grown in light. This picture shows that more, longer and thinner new roots were formed. The longest roots had a length of ± 2 cm. There was also less callus on the root explants.

Figure 32: New adventitious roots formed in liquid ½ nitrate MS medium placed in darkness (5 weeks)

Figure 33: New adventitious roots formed in liquid ½ nitrate MS medium placed in light (5 weeks)

IBA concentration

- After 1 week, callus was visible on the root explants in 4 mg/L IBA. On the explants placed in 2 mg/L IBA no callus was formed and a lot of new roots were growing. When the explants were placed in auxin free medium in the darkness, some new roots of ± 0,3 cm were visible after 1 week.
- 2 weeks in culture yields a lot of adventitious roots when supplemented with 2 mg/L IBA. In the medium with 4 mg/L IBA the medium became cloudy because of the callus. But a lot of small adventitious roots were visible. A auxin free medium afforded a small amount of long adventitious roots.
- The results after 3 weeks are showed in figure 34. A few long roots (± 2 cm) developed on auxin free medium. The concentration of 2 mg/L IBA gave a lot more but shorter adventitious roots. The ones formed in 4 mg/L IBA produced even shorter roots with more callus. These results were comparable with the results of Distave (1998).

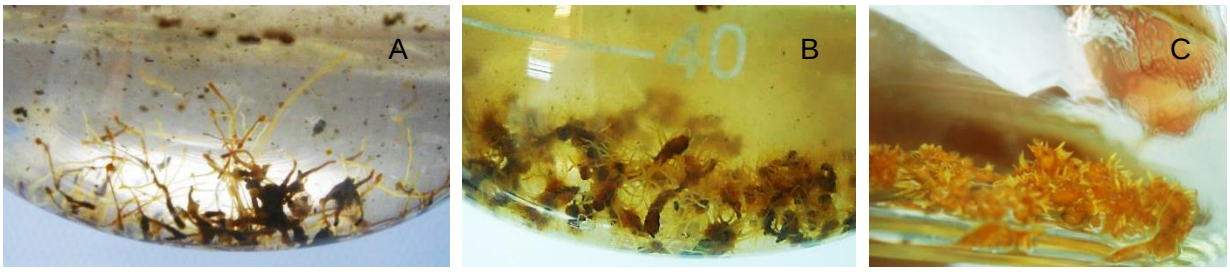

Figure 34: Comparison between root explants in liquid ½ nitrate MS medium supplemented with 0 mg/L IBA placed in darkness (A), 2 mg/L IBA in light (B) and 4 mg/L IBA the light(C) (3 weeks)

IAA concentration

After 2 weeks no callus was formed. Only new roots were growing (Figure 35A). Four weeks in culture afforded a good amount of adventitious roots without callus formation or becoming darker (Figure 35B).

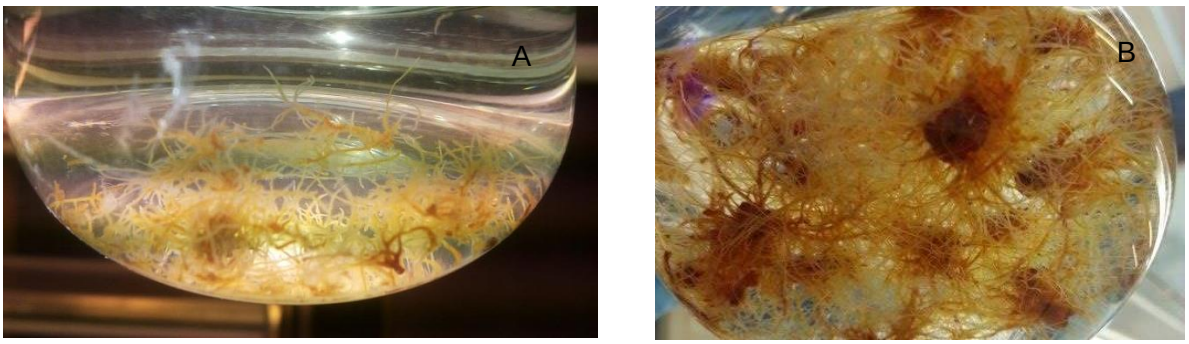

Figure 35: Root explants in liquid ½ nitrate MS medium supplemented with 2 mg/L IBA after 2 weeks (A) and after 4 weeks (B)

3.8.4. Conclusion

Out of the first experiment can be concluded that the multiplication of adventitious roots goes better when the explants are placed in the light. More and longer new roots are formed after 5 weeks with less callus production.

The second experiment makes clear that 2 mg/L IBA is the most suitable concentration to induce more adventitious roots in a shorter time without the formation of callus. Already after 2 weeks a lot of new roots are visible.

Only the concentration 2 mg/L was tested for the auxin IAA. It gave a good amount of roots after 4 weeks. No callus was growing on the root explants and the roots remained light in colour.

Out of these experiment can be concluded that a ½ nitrate MS medium supplemented with 2 mg/L IAA placed in the light is the most suitable condition out of these treatments for the multiplication of adventitious roots in liquid medium.

3.9. Multiplication of adventitious roots in a small bioreactor

3.9.1. Introduction

In the beginning not enough isolated roots of *Pentas lanceolata* were present to set up an air-lift bioreactor. So the purpose of this experiment was to test, with a small amount of roots in small bioreactor, if it is possible to multiply the isolated roots in a bioreactor.

3.9.2. Specific materials and methods

First the small bioreactor was designed as describes in point 2.6.4. with an Erlenmeyer of 250 ml and tested with 100 ml water. To prevent losing roots of *Pentas lanceolata*, isolated roots of *Panax ginseng* and leaves of *Pentas lanceolata* were transferred into the small bioreactor. Later another design was made. In this design the Erlenmeyer was replaced by a Duran bottle. Figure 36 illustrates the two designs of the small bioreactor.

Figure 36: Small bioreactor made of an Erlenmeyer and a Duran bottle

3.9.3. Results and discussion

The small bioreactor made of an Erlenmeyer with 100 ml medium seemed not so suitable. The explants stuck on the wall of the Erlenmeyer and after a few days the medium level decreased. To prevent the losses of medium, the Duran bottle was used. To prevent the roots from sticking on the wall of the small bioreactor, more medium was added. But still in both cases there was no improvement.

3.9.4. Conclusion

Because of the quickly evaporation of the medium, it was better not to transfer the roots of *Pentas lanceolata* into these small bioreactors.

3.10. Multiplication of adventitious roots in the air-lift bioreactor

3.10.1. Introduction

The purpose of this experiment is to set up an air-lift bioreactor with *Pentas lanceolata* to multiply the adventitious roots on large-scale. A lot of optimization needed to be done for this step, therefore a lot of isolated roots was required. Due to the lack of enough isolated roots, the optimal auxin concentration found in earlier experiments on small-scale was used. In this experiment the parameter 'inoculation density' was tested.

3.10.2. Specific materials and methods

Preparation

First the explants were prepared by weighing and cutting the fresh roots. To weigh the roots, a sterile balance was placed in the laminar flow (Figure 37). In this experiment the four inoculation densities 6 g/L, 7 g/L, 8 g/L and 10 g/L were tested. So these amounts were placed in sterile plastic containers and stored in the refrigerator. The materials and methods to set up the air-lift bioreactor are described in point 2.6.5 of the general materials and methods.

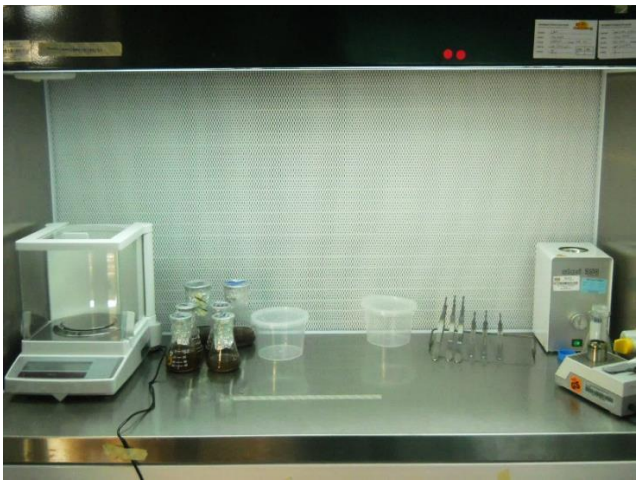

Figure 37: Preparation of the explants in the laminar flow

Treatment

When the bioreactor was autoclaved, the weighed roots were transferred together with the liquid ½ nitrate MS medium supplemented with 2 mg/L IBA into the bioreactor vessel. The method for the inoculation is described in point 2.6.5.

Two bioreactors of 3 L were filled with 2 L medium. In one bioreactor 14 g fresh roots were initiated, so this gives an inoculation density of 7 g/L. The other one was initiated with 16 g fresh roots, this makes an inoculation density of 8 g/L. Also two bioreactors of 5 L were used. One bioreactor was filled with 4 L medium and 24 g fresh roots, which gave an inoculation

density of 6 g/L. The other 5 L bioreactor was filled with only 3 L and 30 g fresh roots. The inoculation density of this last bioreactor was 10 g/L. All the bioreactors were placed in the culture room with a temperature of 22±2 °C and 16 h photoperiod. After four weeks the roots were subcultured in a bigger bioreactor.

Assessment

Every week the growth of the roots was observed and after 8 weeks the roots were harvested and the yield was determined.

3.10.3. Results and discussion

After 1 week of culture in the bioreactor, already small adventitious roots were formed in the bioreactor with an inoculation density of 7 g/L. Overall the colour of the roots turned darker brown and the medium became light brown (Figure 38). Perhaps this colour change is a result of phenol production. One of the four bioreactors (8 g/L) was contaminated after 1,5 week (Figure 39). Clear yellow balls around the roots are growing. Probably during weighing or transferring the explant has become contaminated with fungi.

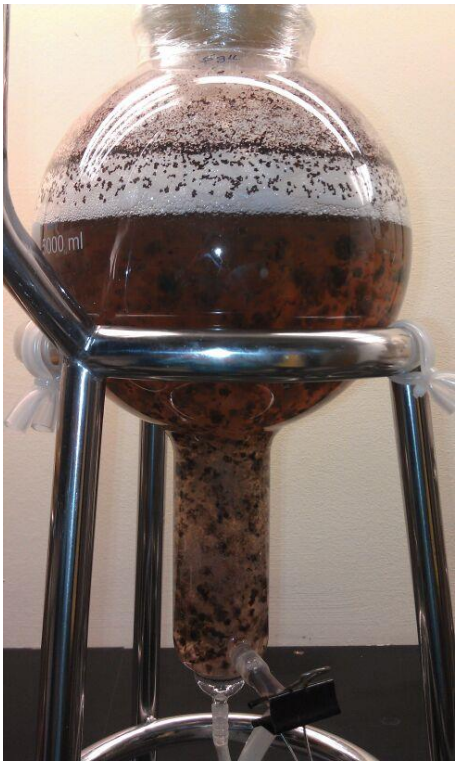

Figure 38: Air-lift bioreactor (6 g/L) after 1 week

Figure 39: Contaminated air-lift bioreactor

One more bioreactor (6 g/L) was contaminated after 2 weeks. In the other two bioreactors a lot of dark callus was growing. The next weeks, adventitious roots started to grow very fast but they still had a dark colour.

After 4 weeks only the bioreactor of 3 L with a inoculation density of 7 g/L was not contaminated. The roots increased in volume, so they were transferred to an air-lift bioreactor of 5 L. The hormone concentration was changed to 5 mg/L IBA. The roots were growing again very fast and the volume increased again. After 3 weeks in the 5 L air-lift bioreactor, the yield amounted 353 g fresh roots. The roots stuck together in dark clusters and short roots with a lighter colour were visible on the outside of the clusters (Figure 40).

In the optimal treatment of Distave (1998) (2 mg/L IBA) the root mass increased 4 times in 4 weeks of shake flask culture. The results of this experiment gave biomass increase of 25 times after 8 weeks culture in the air-lift bioreactor. So the multiplication of these adventitious roots in the air-lift bioreactor produced more roots in the same period.

Figure 40: Harvested roots

3.10.4. Conclusion

Because there are only results of the inoculation density 7 g/L, nothing can be said about the optimal density. Out of this experiment can be concludes that it is possible to produce adventitious roots of *Pentas lanceolata* in the air-lift bioreactor. From the initial weight of 14 g roots, 353 g roots can be harvested after 7 weeks. This is twenty-five times more than the original weight and more effective than the shake flask culture.

Still too much callus is growing in the first weeks of culture in the bioreactor. More research need to be done to find the optimal medium and treatment. Again the inoculation density should be tested. Also the optimal auxin concentration, the percentage of sugar, the air temperature and the length of the explants are parameter which should be established.

3.11. Shoot regeneration from adventitious roots

3.11.1. Introduction

If the production of adventitious roots would be optimized, it would be interesting if shoots can regenerate out of the adventitious roots. In this experiment the adventitious roots were first placed for 5 days on callus-inducing medium (CIM) before transferring them to shoot inducing-medium (SIM) (Valvekens *et al*.,1988). The purpose of this experiment was to check if it is possible the induce shoot meristems and maybe later further research can follow.

3.11.2. Specific materials and methods

Medium

The CIM contains MS medium supplemented with auxin 2,2 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and cytokinin 0,2 µM kinetin. Four different SIM were tested. The SIM contains MS medium supplemented with 0,86 µM IAA and the cytokinins 25 µM N6-2-isopentenyl (2iP), meta-fluorotopolin (mF9R), phenyl adenine (PA) or thidiazuron (TDZ).
The method for the use of petri dishes is described in point 2.6.2.

Inoculation explant

Roots of *in vitro* shoots were cut in pieces of approx. 1-2 cm and initiated on the semi-solid medium. For each treatment, 4 petri dishes with 9 root explants were used. The petri dishes were placed in the culture room with a temperature of 22±2 °C and 16 h photoperiod. First the roots were placed for 5 days on CIM followed by 5 weeks on SIM.

Assessment

All the root explants were observed at 2 and 5 weeks.

3.11.3. Results and discussion

After 2 weeks on the SIM, some green callus spots were visible in the treatments with the cytokinins mF9R, 2iP and TDZ. The roots on PA supplemented medium only produced light yellow coloured callus. The treatment with TDZ caused more callus production. These callus had an soft look. Between this soft callus some green spots were viewable (Figure 41). When mF9R was used, there was less callus production and the roots were brown. On the dark roots green and yellow callus bulbs were growing (Figure 42). 2iP gave the same result, but more callus bulbs were growing all over the roots (Figure 43).

The treatment with PA showed after 5 weeks a lot of dense green callus over the whole root. On some places dark orange soft callus with very small roots were growing (Figure 44). On some explants small light green callus nodules were growing (Figure 45). This was maybe

the beginning of a shoot meristem. The treatment with TDZ gave almost the same result, but more aqueous callus with very small roots was growing. On mF9R most of the explants were black in colour and small amounts of green callus were growing in nodules (Figure 46). On 2iP supplemented medium the green callus bulbs became bigger and a little bit of orange callus was growing in between. Adventitious shoots did not develop.

Figure 41: Root explant on SIM supplemented with TDZ (2 weeks)

Figure 42: Root explant on SIM supplemented with mF9R (2 weeks)

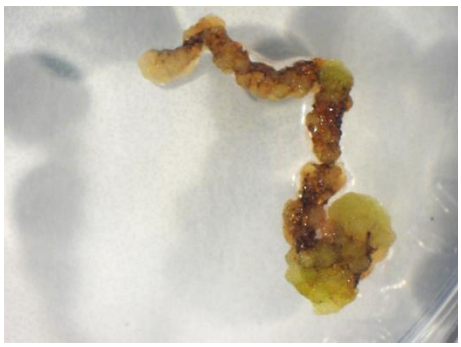

Figure 43: Root explant on SIM supplemented with 2iP (2 weeks)

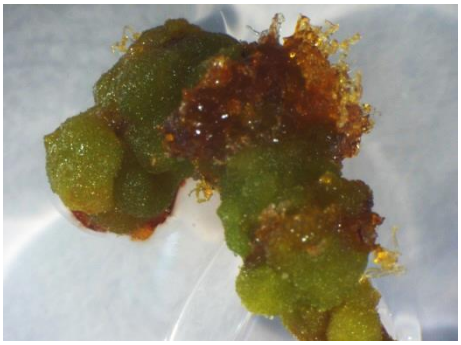

Figure 44: Root explant on SIM supplemented with PA (5 weeks)

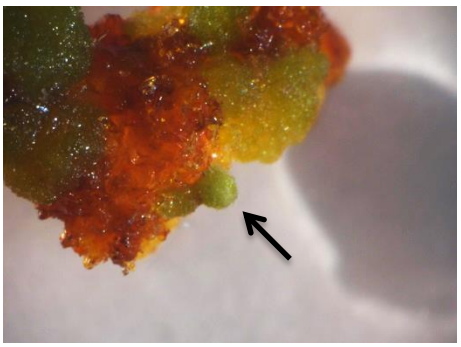

Figure 45: Root explant on SIM supplemented with PA with light green callus nodule (5 weeks)

Figure 46: Root explant on SIM supplemented with mF9R (5 weeks)

3.11.4. Conclusion

In this experiment no clear shoot meristems were formed. But the different cytokinins gave a different result and produced different types of callus. There was white, green, orange, dense and soft callus on the root explants. In general mF9R induced the least green callus. 2ip, TDZ and PA induced more green callus and TDZ and PA also caused a dark orange soft callus on top of the green dense callus. TDZ and PA are probably the most suitable because small light green callus nodules were visible on the root explants. Maybe these can grow out as a shoot meristem.

3.12. Shoot multiplication

3.12.1. Introduction

The easy flowering was a remarkable observation during the initiation and micropropagation of the *Pentas lanceolata* shoots. In addition to the fact that it is a beautiful ornamental, this makes the plant more interesting for shoot multiplication. The multiplication of shoots can go faster by using a bioreactor. The uptake of the nutrients goes more efficient and less labor is required (McAlister *et al*., 2005). In these experiments different bioreactors are tested to multiply numerous shoots in a shorter period. To test if the shoots are suitable for a culture in liquid medium, the shoots were first placed in a shake flask. Later the RITA®, the SETIS™ and the air-lift bioreactor were examined.

3.12.2. Specific materials and methods

Explant

Shoots, grown in semi-solid medium, with an average length of 4 cm were used as explant. All bioreactors were placed in the culture room with a temperature of 22±2 °C and 16 h photoperiod.

Shake flask

The method for the use of the shake flask was described in point 2.6.3. One difference is that the shoots were placed in a thin layer of medium. Full MS medium supplemented with 0,45 mg/L BA was used.

RITA®

The materials and methods about the RITA® are retrievable in point 2.6.7. Full MS medium supplemented with 0,45 mg/L BA was used and every 8 hours the medium flowed into the bioreactor vessel.

SETIS™

The materials and methods about the SETIS™ are retrievable in point 2.6.8. Full MS medium supplemented with 0,45 mg/L BA and full MS medium supplemented with 1,20 mg/L mTR were examined. Every 8 hours the medium flowed into the bioreactor vessel.

Air-lift bioreactor

With the purpose to apply it later with *Pentas lanceolata*, only shoot of *Ananas comosus* were initiated in the air-lift bioreactor. The medium for *Ananas comosus* is already optimized. The same air-lift bioreactor, as illustrated in figure 3, was used. In section 2.6.5. the total set up of the air-lift bioreactor is illustrated. A small difference in the set-up of the bioreactor was the netting. The netting was glued horizontally in the middle of the vessel and the vessel was

filled till the liquid medium reaches a height of 1 cm above the netting. Due to this netting the shoots cannot whirl in the medium but rest at the netting. It was necessary to refill the medium when the level of the medium was decreased under the netting.

3.12.3. Results and discussion

Shake flask

All five shoots still looked beautiful green after 1 week in the shake flask. On the cutting site of one shoot small roots were growing. After 3 weeks the leaves became larger and they still had a green colour. On one shoot one flower bud was visible (Figure 47). The roots on that one shoots became longer.

The shoots showed the same result as in the semi-solid medium. Green leaves enlarged, roots were growing and again flowers buds were visible.

Figure 47: Shoot in shake flask with flower bud (3 weeks)

RITA®

In the first attempts the medium became cloudy after 3 days. Later the shoots turned brown. Probably the medium was contaminated during the replacement from the sterilization bottle to the RITA®. In the next attempts the medium was already sterilized in the RITA®. So only the shoots needed to be transferred. This technique resulted in 1/3 sterile bioreactors. This is still not good enough and there is a risk that the medium will destroy the air filters by boiling during the sterilization.

With this method a lot of shoots were wasted. It is very important that the bioreactor is clean and sterile. The bioreactor need to be cleaned with soap and ethanol before sterilization. All the openings have to be sealed with polyethylene foil and the bioreactor has to be sterilized in a closed sterilization bag. Directly after sterilizing, they need to be placed in the laminar flow. Sterilization and initiation should be done on the same day to avoid contamination after sterilization. Experience in initiating of the bioreactor can be an advantage.

SETIS™

After three weeks the shoots had more and bigger leaves. More new shoots were formed in the SETIS™ with mTR supplemented medium. Also roots were growing on the end of the shoots. In the case with BA as cytokinin, no roots were noticeable and the shoots were smaller. Three weeks later the SETIS™ with mTR was contaminated. The shoots in the BA supplemented medium produced also bushes of roots (Figure 48). The old leaves had a browner colour but new fresh green leaves were growing.

Also isolated roots were transferred in the SETIS™. This experiment failed due to contamination.

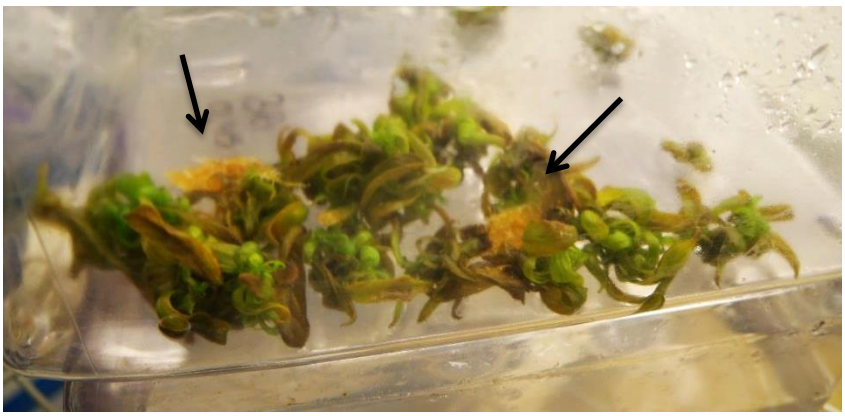

Figure 48: Shoots in SETIS™ with 0,45 mg/L BA supplemented medium with bushes of roots (6 weeks)

Air-lift bioreactor

Every week the shoots became larger and a clear increase of shoots number was visible (Figure 49). After 3 weeks the medium decreased under the netting. Probably this method is suitable for the multiplication of *Pentas lanceolata.*

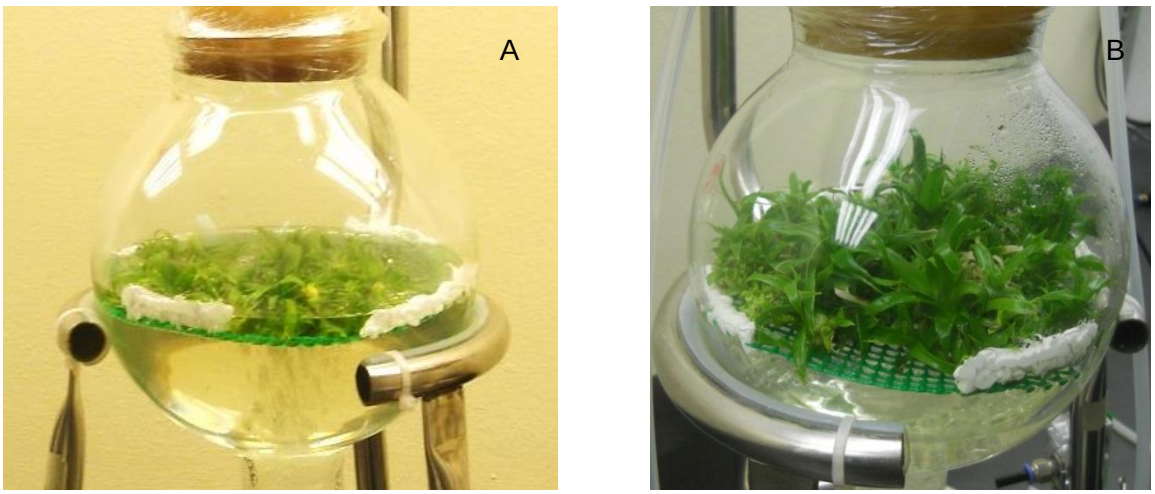

Figure 49: Shoots of *Ananas comosus* in the air-lift bioreactor initial (A) and 3 weeks in culture (B)

3.12.4. Conclusion

These experiments made clear that the shoots can grow in liquid medium but well trained people are required to handle these bioreactors. The air-lift bioreactor was not contaminated due to the experience during the adventitious root culture.

In the experiment with the SETIS™ is seen that the cytokinin mTR gives better results. After a cultivation of 3 weeks, the shoots were larger and more new shoots were formed in comparison with the treatment of BA.

All these experiments need to be repeated to determine the optimal treatment for the multiplication of *Pentas lanceolata* shoots in bioreactors.

General conclusion

Adventitious root induction

The first purpose of this work was to determine the best protocol to induce adventitious roots of *Pentas lanceolata*. In general, all the experiments confirmed that a higher induction of the adventitious roots can be obtained when the explants are placed in the light and leaf explants results in a higher amount of newly formed roots compared to root explants. The roots induced on leaf explants are also longer and contain less callus.

For both auxins, IBA and IAA, lower concentrations cause more adventitious roots from leaf explants. IAA is more potent for the induction of adventitious roots. After 4 weeks in culture the treatment 2 mg/L IBA caused a mean of 6,50 adventitious roots per leaf explant, while the treatment 2 mg/L IAA resulted in a mean of 24,25 adventitious roots. Also the mean length of the roots on 2 mg/L IAA was higher. The experiment with the lower concentrations made clear that 1 mg/L IAA and IBA induced even more adventitious roots. Again the auxin IAA is the most suitable. When the concentration increases, the number of the adventitious roots decreases and the amount of callus increases.

In the experiments with root explants, different concentrations of the auxins IBA, IAA and also NAA were tested. NAA seemed not suitable for the induction of new roots on *Pentas lanceolata.* The most suitable IBA concentration was 2 mg/L with a mean of 8,07 new roots. So root explants react better on IBA than leaf explants. With a mean of 13,85 new roots, the concentration 10 mg/L IAA induced the most new roots of all treatments on root explants. Also lower concentrations of IAA and IBA were tested on root explants. Out of these experiments can be concluded that lower concentrations are also suitable for root explants. Earlier was concluded that 10 mg/L IAA was more suitable compared to 2 mg/L IBA. But 1 mg/L IBA gave a visible better result than 10 mg/L IAA. When the concentration increases, the number and length of the roots decreases. So root explants on medium supplemented with 1 mg/L IBA and placed in the light, affords the most new roots on root explants. Nevertheless leaf explants placed on medium supplemented with 1 mg/L IAA in the light, affords the most and longest new roots compared to all the other tested treatments.

Out of the results of the acute gamma irradiation test can be concluded that 47 Gy reduces the number of new roots on root explants of *Pentas lanceolata* to 50%. To be sure about this conclusion, this test need to be repeated with the optimal root induction medium.

Adventitious root multiplication

The second purpose was trying to further multiply the adventitious roots in the air-lift bioreactor. The multiplication was first tested in liquid medium in a shake flask culture. Also the lower concentrations of auxins were more suitable for the multiplication of the adventitious roots in liquid medium. The concentration 2 mg/L IBA gave a better result than 4 mg/L IBA. The result of treatment 2 mg/L IAA gave even better results compared to the IBA treatment. No callus was growing on the root explants and the roots remained light in colour. The induction of roots from leaf explants in liquid medium was very low. Maybe IAA need to be tested instead of IBA.

Different inoculation densities were inoculated in the air-lift bioreactor and the medium was supplemented with 2 mg/L IBA. This auxin was chosen because the induction of new roots on root explants with IBA gave good results. But no conclusion can be made about the optimal inoculation density. The only air-lift bioreactor without contamination was inoculated with 7 g/L roots. This treatment seemed suitable for the multiplication of adventitious roots of *Pentas lanceolata*. After 7 weeks the biomass was increased from 14 g to 353 g fresh roots. But still too much callus was growing in the first weeks of culture in the bioreactor. More research need to be done to find the optimal medium and treatment. The inoculation density and lower auxin concentration should be tested. Also the percentage of sugar, the air temperature and the length of the explants are parameters which should be determined.

Shoot cultures

The last purpose was to regenerate shoots from these adventitious roots in order to obtain a beautiful flowering ornamental plant again. During this experiment no clear shoot meristems were formed. But the four different cytokinins gave a different result and produced different types of callus. The cytokinins TDZ and PA are probably the most suitable because small light green callus nodules were visible on the root explants. Maybe these can grow out as a shoot meristem. Further research need to be done to find the best treatment for shoot regeneration from root explants of *Pentas lanceolata*.

The experiments with the temporary immersion systems made clear that the shoots can grow in liquid medium but well trained people are required to handle these bioreactors. The cytokinin mTR gave better results in the SETIS™. After a cultivation of 3 weeks, the shoots were larger and more new shoots were formed compared to the treatment with cytokinin BA.

Next to the results of interest, it was observed that *Pentas lanceolata* flowers exceptionally well *in vitro* without using specific treatment for flowering. The basic medium supplemented with BA or mTR gave no different results. This observation makes this plant more interesting for further research.

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Appendix

1. Compound of ½ nitrate MS medium

Stock solution	Component	Quantity
Stock solution 1: Sulphate	$MgSO_4.7H_2O$	18,5 g
	$MnSO_4.H_2O$	0,84 g
	$ZnSO_4.7H_2O$	0,43 g
	$CuSO_4.H_2O$	0,00125 g
Stock solution 2: Nitrate	NH_4NO_3	82,5 g
	KNO_3	95 g
Stock solution 3: Halide	$CaCl_2.2H_2O$	22 g
	KI	0,0415 g
	$CoCl_2.6H_2O$	0,00125 g
Stock solution 4: Phosphate	KH_2PO_4	8,5 g
	H_3BO_3	0,373 g
	$Na_2MoO_4.2H_2O$	0,0125 g
Stock solution 5: FeEDTA	$C_{10}H_{12}N_2O_8FeNa$ (EDTA Ferric sodium)	3,73 g
Stock solution 6: Vitamins	Myo-Inositol	10 g
	Thiamin HCl	0,01 g
	Nicotinic Acid	0,05 g
	Glycine	0,2 g
	Pyridoxine	0,05 g

Component	Quantity
Distilled water	1000 ml
Stock solution 1, 3, 4	10 ml
Stock solution 2, 5, 6	5 ml
Sucrose	50 g
Gelrite	2,3 g