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Studies of root formation of micropropagated shoots *in vitro* and cuttings from light treated mother plants *ex vitro* of Manchurian Dutchman's pipe (*Aristolochia manshuriensis*)

Studier av rotbildning hos mikroförökade skott *in vitro* och sticklingar av ljusbehandlade moderplantor *ex vitro* av koreansk pipranka (*Aristolochia manshuriensis*)

Master thesis
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Abstract

The ornamental plant *Aristolochia manshuriensis* is difficult to propagate by cuttings and therefore a propagation method *in vitro* has been developed. However the published method has several limitations due to unpredictable and low rooting percentage as well as low survival rate.

In this study several factors were investigated in order to improve rooting *in vitro* and survival *ex vitro*. To improve rooting the amount of cytokinin was reduced before rooting. Different types and levels of auxins and the use of riboflavin together with indole-acetic acid (IAA) were tested. In order to change the ratio between carbon and nitrogen, different concentrations of macro nutrients were used. Glutamine as the only nitrogen source was tested as well as different types of iron source. Addition of activated charcoal to the hormone free medium in combination with long or short exposure to auxin were used to improve both rooting and survival.

In order to improve rooting of cuttings *ex vitro*, the mother plants were grown under different light regimes (20 and 200µmol m^{-2} s^{-1}) and cuttings were treated with different type and concentrations of auxin with and without glucose.

The rooting process for the cuttings taken *ex vitro* was very slow and good rooting was in some cases not obtained until after 34 weeks. After 9 weeks the highest rooting frequency was only 40%. The cuttings have a tendency to stay green during long time, even without callus or root formation. In some cases the shoot produced new leaves without any production of roots.

Mother plants grown at 20 µmol m^{-2} s^{-1} were more vigorous and the cuttings from them had better rooting percent than from those grown under 200µmol m^{-2} s^{-1}. The best rooting was 70% for cuttings treated with IAA at 500 mg/l after 34 weeks. In general the treatments with auxins and sugar gave the lowest rooting capacity.

Rooting *in vitro* of microshoots resulted in more rapid rooting. After 3 weeks, on medium containing 1/3 of macro nutrients and IAA at 20 mg/l, 52% rooting was obtained. However these plantlets were not transferred to *ex vitro*.

The most rapid *in vitro* rooting was obtained after 2 weeks using dipping in IBA solution at 250 mg/l for 30 minutes and then transferred to hormone free medium containing 10g/l activated charcoal resulting in 24% rooting. After planting in soil, 60% rooting was recorded after 13 weeks.

Several months after the *in vitro* rooting experiments had been planted *ex vitro* there were explants thought to be dead but when examining them thoroughly callus was found growing from the root lump or new leaves and roots under the surface. Treatments resulting in high callus formation had lower rooting than treatments with less callus formation.

Abbreviations

BAP = Benzylaminopurine; **IAA** = Indole-3-acetic acid; **IBA**= Indole-3-butyric acid; **NAA** = Naphthalene acetic acid; **Lepoivre** = Quoirin et al. (1977); **MS** = Murashige and Skoog (1962); **AC** = Activated charcoal; **Gr.** = Group; **w.** = weeks

Sammanfattning

Prydnadsväxten *Aristolochia manshuriensis* är svår att sticklingsföröka och därför har en *in vitro* förökningsmetod utvecklats. Denna publicerade metod har emellertid många begränsningar beroende av låg och ojämn rotbildning såväl som låg överlevnad.

I denna studie har flera faktorer undersökts för att förbättra rotning *in vitro* och överlevnad *ex vitro*. För att förbättra rotningen reducerades cytokininhalten före rotinduceringen. Olika typer och koncentrationer av auxin testades och även användning av riboflavin tillsammans med indolättiksyra (IAA). För att ändra kol och kvävekvoten, användes olika koncentrationer av makronäringsämnen. Glutamin tillfördes som enda kvävekälla och olika järnkällor utvärderades. Tillsats av aktivt kol i det hormonfria mediet i kombination med lång eller kort exponering av auxin testades för att förbättra både rotning och överlevnad.

För att förbättra rotning hos sticklingar tagna *ex vitro*, odlades moderplantor under olika ljusintensiteter (20 och 200µmol m^{-2} s^{-1}) och dess sticklingar behandlades med olika slags auxin i olika koncentrationer med och utan tillsats av glukos.

Rotningsprocessen för sticklingar tagna *ex vitro* var långsam och god rotning uppnåddes i vissa fall inte förrän efter 34 veckor. Efter 9 veckor var den bäst uppnådda rotningen endast 40%. Sticklingarna hade en tendens att förbli gröna under lång tid, även utan kallusbildning eller rotbildning. I vissa fall kunde skotten bilda nya blad utan att ha bildat rötter.

Moderplantor odlade i 20 µmol m^{-2} s^{-1} var vid bättre vigör och sticklingarna från dessa moderplantor gav bättre rotningsprocent än från dem som odlades i 200µmol m^{-2} s^{-1}. Den bästa rotningen var 70% för sticklingar behandlade med IAA 500 mg/l efter 34 veckor. Generellt sett hade sticklingar behandlade med auxin och socker sämst rotningskapacitet.

In vitro-rotning av mikroskott resulterade i snabbare rotning. Efter 3 veckor, på medium innehållande 1/3 makronäringsämnen och IAA 20 mg/l, uppnåddes 52% rotning. Dessa explantat överfördes inte *ex vitro*.

Den snabbaste *in vitro* rotningen uppnåddes efter 2 veckor med doppning i 250 mg/l IBA lösning i 30 minuter och sedan överförda till hormonfritt medium innehållande 10g/l aktivt kol vilket resulterade i 24% rotning. Efter plantering i jord, noterades 60% rotning efter 13 veckor.

Flera månader efter rotningsförsöken *in vitro* planterats ut *ex vitro* fanns det explantat som verkade döda men som vid närmare undersökning visade sig ha kallus växandes vid rotklumpen eller nya blad och rötter under jorden. De behandlingar som gav mycket kallusbildning hade sämre rotning än behandlingar med mindre kallusbildning.

Förkortningar

BAP = Benzylaminopurine; **IAA** = Indolättiksyra; **IBA**= Indolsmörsyra; **NAA** = Naftylättiksyra; **Lepoivre** = Quoirin et al. (1977); **MS** = Murashige och Skoog (1962); **AC** = Aktivt kol; **Gr.** = Grupp; **w.** = veckor

1 Introduction

Around 80% of all living plant species on earth are Angiosperms, flowering plants. Within its subclass dicotyledons we find the superorder *Mangoliids*. Many of the features within the *Mangoliids* have primitive impressions (Dahlgren and Björkqvist, 1993). *Mangoliids* contain the four orders *Magnoliales, Laurales, Canellales* and *Piperales*. Within the order *Piperales* there are five families of herbaceous or somewhat woody species with ethereal oil cells and often with alkaloids. One of the families is *Aristolochiaceae* which contains 600 species in total, 500 being *Aristolochia*-species including woody vines or herbaceous perennials from which many are toxic and medically used. They are commonly called dutchman's pipe or pelican flower. (Bremer et al., 2003)

1.2 Nomenclature

Aristolochia derive from the Greek words 'aristos'- best or the best, and 'locheia' - child bearing (Corneliuson, 2000). The name manshuriensis means that it originates from the province Manchuria in China.

The botanical name in Latin is *Aristolochia manshuriensis* (Aldén et al., 1998; Svensk kulturväxtdatabas, http://skud.ngb.se) but it's easy to find rare spellings as *A. man**ch**uriensis, A. man**sch**uriensis, A. man**dsh**uriensis* and *A. man**dsch**uriensis* in scientific texts.

Aristolochia manshuriensis (Komarov) is synonymous with *Hocquartia manshuriensis* (Komarov) and *Isotrema manchuriensis* (Komarov) (Aldén et al., 1998; Ministry of health Malaysia, www.bpfk.gov.my).

The Swedish name is koreansk pipranka or manchurisk pipranka (Aldén et al., 1998; Svensk kulturväxtdatabas, http://skud.ngb.se).

1.3 Morphology of Aristolochia manshuriensis

The Flora of China (2003) describes the woody liana *Aristolochia manshuriensis* as a climbing shrub with slender stems, marked with fine longitudinal lines or ridges. The petioles are about 6-8 cm. The leaves are leathery and light green in color, abaxially covered with dense white hairs. They are fairly heart-shaped with the point away from the stem; the size is in the region of 15-30 cm in diameter. They are palmate veined and the principal veins arise from the end of the petiole and radiate towards the edge of the leaf.

The flowers are either single or paired, situated in axils. The peduncle is about 3 cm long and it is pendulous and glabrous. The bracteoles are inserted below the middle of the peduncle (Gardenweb botanical terms, http://glossary.gardenweb.com/glossary). They are about 1 cm and cordate in shape (Flora of China, 2003; www.efloras.org). The tube-like flowers are yellow with a purple edge and about 5 cm (Lagerström, 1998). They are swollen at the base and ends at the top with a hood, in between they are curved (Burnie, 1999). The curved part is 4.5 – 5.5 cm long and greenish-yellow. The limb of the petal is disc-like and three lobed, 4-6 cm in diameter and purple reddish-brown (Lagerström, 1998). These lobes are broadly deltoid as a low trian-

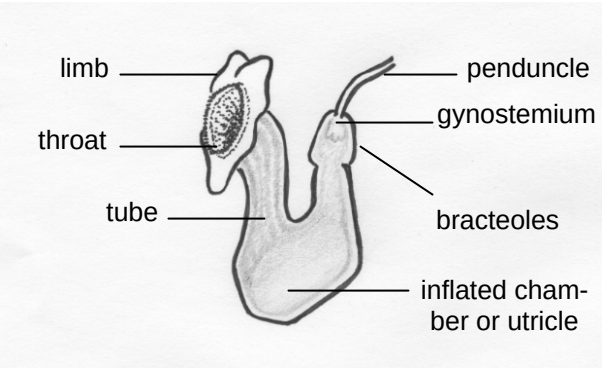

Figure 1.1: The flower of *Aristolochia manshuriensis*

gle attached at the middle of its wide part, resembling the Greek letter delta (Gardenweb botanical terms, http://glossary.gardenweb.com/glossary).

The flowers functions as flytraps. Insects are tempted to enter the long tube of the flower by its strong smell. The insect is often kept inside the flower until the anthers have maturated (Bremer et al., 2003) and powders it with pollen (Burnie, 1999).

The fruit is a green, narrowly cylindrical capsule, 9-11 cm long and 3-4 cm in diameter (Flora of China, 2003; www.efloras.org).When mature, the fruit opens basipetally and incompletely, forming a hanging basket from which the wind-dispersed seeds are released (Bremer et al., 2003; Burnie, 1999). The seeds are about 0.6 cm in diameter with both surfaces slightly convex and verrucose (Flora of China, 2003; www.efloras.org).

In China, *Aristolochia manshuriensis* flowers from June to July and it sets fruit in August to September (Flora of China, 2003; www.efloras.org).

1.4 Growth of Aristolochia under natural and Swedish conditions

The ornamental plant *Aristolochia manshuriensis* was introduced in Sweden 1976 as the Nordic Arboretum committee realized a collecting expedition to South Korea. *A. manshuriensis* were brought to Sweden as seeds, which were later cultivated at SLU in Ultuna (Lagerström, 1998).

We have two hardy species of the genus *Aristolochia* in Sweden. From North America comes *Aristolochia macrophylla* (*syn. A. durior or A. sipho*), which is closely related to *Aristolochia manshuriensis*. The differences noted for *A. manshuriensis* are faster and stronger growth, bigger leaves and bigger, slightly different flowers than *A. macrophylla*. Another notable difference is that the new wines on *A. manshuriensis* are hairy, light green and already covered with corky wings the second year of growth, meanwhile the wines of *A. macrophylla* stay dark green longer. Lagerström (1998) have found *A. manshuriensis* to be quite hardy and it can grow in the northern parts of Sweden, at least to zone V. According to Rångedala plantskola (www.rangedala-plantskola.se) its relative *A. macrophylla* is hardy to zone III.

According to Lagerström (1998) the best development of *A. manshuriensis* is achieved in soil rich of nutritions and organic matter. It grows well both in sun and in shadow, but a warmer and well-drained site improves the hardiness (Lagerström, 1998). The further north it's growing, the more important it is to cultivate it in its appropriate site. In China it can be found in Gansu, Heilongjiang, Hubei, Jilin, Liaoning, Shaanxi, Shanxi and Sichuan in mixed forests within moist shady areas, some 100-2200 meters over sea level. (Flora of China, 2003; www.efloras.org)

It's a very good high climber on facades since it grows vigorous and fast. A path covered with *A. manshuriensis* as an arched leaf path, will give quite an appealing shadow movement. This way the interesting flowers which normally are very well hidden among the big leaves come into eyesight. (Lagerström, 1998)

1.5 Propagation of Aristolochia-species in Sweden

As a general rule, shrubs and woody vines are propagated by cuttings (Hartmann et al., 2002). Both for propagation by cuttings as for micropropagation the rooting ability is the limiting factor who determines which species are economically interesting to propagate (Welander, 1995).

According to Hansen (1999) the best method for propagation of *Aristolochia*-species would be layering, in second hand herbal base cuttings and last propagation by seeds. Hansen (1999) also notes that the seeds do not need any treatment before germination. This is probably writ-

ten with *A. macrophylla* in mind, since *A. manshuriensis* is relatively new on the market. Until 1998, there was no known report of any fruits being developed in Sweden and therefore it's very important to develop methods for vegetative propagation other than using seeds (Lagerström, 1998).

1.5.1 E-planta

The E-plant material comes from a clone that grows in Ultuna since 1980. *A. manshuriensis* is micropropagated and delivered to the nurseries as small plants. After growing in the nursery for a season they are ready to be sold. The aim is to get a strong growing plant, similar to the first plants cultivated from seeds in Ultuna. These reached a good size and quality in only one season. After developing a method for micropropagating at Elitplantstationen in Balsgård, this species has now been propagated during more than 17 years. The main reason for producing the genus *Aristolochia* is its fast and luxuriant foliage growth already as a small plant and in particular the species *A. manshuriensis* for its satisfactory hardiness. (Lagerström, 1998; E-planta, www.eplanta.com)

1.5.2 Bioreactors

The costs for micropropagation can for some species be reduced by up to 65% using bioreactors. At SLU Alnarp, a system for micropropagation with small bioreactors has been developed. They are of one litre and contain two chambers, one for the explants and one for the nutrient solution. When flooding the explants the solution is pumped from one chamber to the other and then the solution descent again. (Sandskär, 2003)

For large scale propagation, liquid are preferred over solid nutrient media since the culture generally grow faster, requires less handling and are easier to automate in liquid media. Not all cultures grow well in liquid media and vitrification can be a problem. (Bonga and von Aderkas, 1992)

1.6 Traditional medical use and new compounds

1.6.1 Traditional use

The Greeks, Hypocrites (460-377 B.C.) and Theophrastus (370-285 B.C.) wrote about *A. manshuriensis* as a remedy for facilitating child deliveries. The Romans, Cicero (106-43 B.C.) and Plinius (23-79 A.D.) also wrote that it was used for snakebites since the root has a similar shape. (Corneliuson, 2000)

Later on during the Middle Ages, herb extracts were used as medicine for different health conditions or diseases depending on the shape of the plant, its roots, leaves or its flowers. The flowers of *A. manshuriensis* reminds of a fetus just before being born. (Corneliuson, 2000) There are numerous medicines containing plant extract from *A. manshuriensis*. For example a heart tonic made from dried stem sections is used in Eastern medicine (Bulgakov, 1989).

Since 1950 *A. manshuriensis* have been widely used as constituent of the Chinese herb Mu Tong. In China and other countries, renal failure due to ingestion of large doses of *A. manshuriensis* has been reported while in traditional Chinese herbal texts, no such toxicity has been recorded. (Zhu, 2002)

Aristolochia plants contains aristolochic acids, which according to Li et al. (2004) result in kidney diseases such as urothelial cancer. Therefore it's important to monitor the amount of aristolochic acid in herbal medicines. Some *Aristolochia* species, like *Aristolochia fangchi* have low amounts of the noxious acids, and populations of *A. fangchi* from the Guangdong region don't seem to have them at all.

Traditional medicines containing plant extracts from *A. manshuriensis* are now banned from the market since research indicates carcinogenic and nephrotoxic (toxic to the kidneys) qualities (UN Department of economic and social affairs, www.un.org; Sociedad Española de farmacía hospitalaria, www.sefh.es; Royal pharmaceutical society of Great Britain, www.rpsgb.org.uk; World Health Organization, www.who.int/en).

Aristolochia is an important genus used for traditional medicine in Asia, foremost in China. During the last twenty years, this genus has attracted a great deal of interest and has been the focus of numerous studies, both chemical and pharmacological. It is a rich source of acids unique to this genus, the aristolochic acids. They also contain a lot of terpenoids. The development in the field of phytochemistry for the *Aristolochia*-species is rapid. (Wu, 2004)

1.6.2 New compounds

Wu (2003) found that extracts from the stem of *Aristolochia manshuriensis* contains three new compounds, demethylaristofolin E, aristomanoside and dehydrooxoperezinone. The last mentioned was found to inhibit the replication of HIV, with an EC50 (the drug concentration that provokes a response halfway between baseline and maximum) value of 17.5 µg/ml and a therapeutic index (the therapeutic index of a drug is defined as the ratio of the toxic dose to the therapeutic dose) of 1.43.

1.7 Different factors influencing rooting

To produce roots it is necessary for the cutting to have access to auxin and glucose as energy source (Welander, 1995). The levels of auxin within the cutting can be elevated or it can be added externally (Hartmann et al., 2002). Murai, et al. (1999) found that the rooting percent was high when the sorbitol content in the cuttings was high and therefore 50 % of the cuttings collected in summer rooted and then the percentage decreased to no rooting in October. The rooting ability also depends of the physiological condition of the mother plant, its age, its genotype (Welander, 1995) and the season of the year at which the explants are taken (George, 1993).

1.7.1 Mother plant etiolation ex vitro

The term etiolation is used when mother plants are grown in conditions of heavy shade. A reduction of irradiance level for the mother plants can increase the number of rooted cuttings for species that are difficult to root. Etiolation has a positive effect on the endogenous auxins in the plant, and can reduce the lignin production, thus releasing phenolic metabolites that enhance root initiation. (Hartmann et al., 2002)

In a study with *Scaevola aemula* cultivated at 20 µmol m^{-2} s^{-1} and 200 µmol m^{-2} s^{-1}, top cuttings from mother plants grown at high light intensity, full nutrition and no auxin did not root at all meanwhile the ones given the same treatment and grown at low light intensity had 100% rooting (Welander, 1992). Experiments with node cuttings from *Populus tremula* cultivated for 52 days in 200 µmol m^{-2} s^{-1} and 20 µmol m^{-2} s^{-1} showed the same results (Welander, unpublished).

1.7.2 Different light intensities in vitro

According to an investigation made by Svensson (2000) on *Aristolochia manshuriensis* the lowest light intensity in the study, 20 µmol m^{-2} s^{-1} resulted in the lowest multiplication rate per month and the highest level tested, 80 µmol m^{-2} s^{-1} resulted in the highest multiplication rate per month. The light intensity during multiplication also affected the rooting later.

The maximum percentage of rooted shoots was attained after two subcultures (12 weeks) at light intensity of 80 µmol m^{-2} s^{-1}. A prolonged period on the last multiplication medium before rooting (6 weeks instead of 4 weeks) increased the multiplication rate significantly but affected subsequent rooting negatively, which dropped from 41% to 12%. Darkness during the first one to seven days on the rooting medium, improved the percentage of rooted shoots. It was also beneficial to split the base of shoots of *Aristolochia manshuriensis* prior to rooting.

1.7.3 Cytokinins

Cytokinins can inhibit or delay root formation (Humphries, 1960; Hartmann et al., 2002). Sometimes it takes more than one subculture in cytokinin-free medium to reduce the levels of cytokinin within the tissues. (George, 1993)

1.7.4 Auxins

There are several natural auxins in higher plants, IAA being the most abundant one (Taiz and Zeiger, 1991). IAA is synthesized in leaf primordia, young leaves and developing seeds (Hartmann et al., 2002; Raven et al., 1999).

IBA occurs naturally in some plant species. It has been shown that IBA supplied *in vitro* partly converts to IAA, and can enhance tissue sensitivity for IAA (Hartmann et al., 2002).

IAA and IBA can easily be metabolized within the plant, but not NAA which is a synthetic auxin and subsequently NAA stays longer in the tissue. NAA at high concentration can influence rooting negatively since auxin stimulates root initiation but inhibit root elongation (Hartmann et al., 2002). IAA is added to media in a relatively high concentration since IAA oxidase may be present in the tissue (Dodds and Roberts, 1995) and additionally it is rapidly degraded by light. Cool-white fluorescent tubes have shown to promote the degradation of both IAA and IBA in agar media. (Nissen and Sutter, 1990)

The NAA molecule is very different from IAA but its activity is very similar. At a neutral pH they have the same charge separation with a strong negative charge from the carboxyl group and a weaker positive one in the ring structure. This may be essential for the activity of this auxin. (Hartmann et al., 2002; Taiz and Zeiger, 1991)

High auxin levels promote the formation of both lateral and adventitious roots. Lateral roots originate from cells in the pericycle meanwhile adventitious roots can be initiated in a variety of locations from mature cells that begin to divide and develop into a root apical meristem. (Taiz and Zeiger, 1991)

It has been confirmed that auxin is needed for initiation of adventitious roots on stems and either applied or endogenous auxin is required for the first root initial cells to divide. In general a high auxin and low cytokinin ratio favours formation of adventitious roots. (Hartmann et al., 2002) There are exceptions from the dual requirements for auxin and cytokinin. Some explants may have high endogenous auxin and others require the addition of auxin but not cytokinin. (Dodds and Roberts, 1995) Some observations on natural levels of auxin suggest that low endogenous auxin levels are required for root initiation, but high levels for root growth (George, 1993).

IAA tends to accumulate just above any wound site because of its polar transport, where it promotes the formation of adventitious roots (Taiz and Zeiger, 1991).

1.7.5 Activated charcoal

Activated charcoal can absorb compounds exuded from the tissues in culture or present in the nutrient solution. Root formation can be promoted by activated charcoal adsorbing inhibiting

substances or by excluding light from the medium. (George, 1993) Most probably activated charcoal binds residual cytokinin leached from the shoots to the medium and consequently improving rooting. It can also stabilize the pH. (Pierik, 1997) Different types of activated charcoal have different adsorptive characteristics and pH, dependent on the manufacturing process (Bonga, 1982).

1.7.6 Nitrogen

A high C/N ratio in a cutting benefits root initiation and a low level of nitrogen in the medium have been found to benefit root formation (Hartmann et al., 2002).

The C/N ratio for *Populus tremula* was changed by manipulating the nutrition solution given to the mother plants in combination with different light intensities. Rooting could be improved either by lowering the light intensity or nitrogen concentration in the nutrient solution given to the mother plants. The highest rooting frequency was obtained on cuttings from mother plants grown at low light intensity and given full nutrition. In this experiment, the node cuttings given full nutrition and no auxins grown at 200 μmol m^{-2} s^{-1} did not root at all whilst cuttings treated the same way but grown at 20 μmol m^{-2} s^{-1} had a rooting percentage of 100%. (Welander, unpublished)

Using glutamine as the sole nitrogen source is an interesting possibility to provide amino acids to the medium, since most inorganic nitrogen in culture media first is converted to amino acids, by the plant tissues, before assimilating them into proteins. Some of the effect can also come from altering the pH in the medium. Glutamine elevates the pH partly during autoclaving and partly during the first 14 days thereafter. (George, 1993)

1.7.7 Iron

Total iron is always higher than the available iron, which depends on the pH. Iron is especially deficient in alkaline solutions. One of the most effective ways to remedy iron chlorosis is to add iron chelates, complex organic molecules that bind metal ions and form a stable water-soluble and relatively chemically inert complex. (Janik, 1986)

Iron as iron chelate is less toxic and well suited to be used *in vitro* (Street et al., 1952).

EDTA was discovered in the 1950's and has thereafter been widely used. Combined with iron, it forms FeEDTA (Janik, 1986). FeEDTA and FeEDDHA are absorbed as uncomplexed ions by the plant roots (George, 1993).

The choice of iron chelate in the micropropagation medium can be decisive when propagating plant species that are susceptible to iron deficiency. It seems like Fe-EDDHA is a more photostable chelate than Fe-EDTA, and it can thereby give a higher availability of iron for the *in vitro* cultivated shoots. EDTA itself can have side effects on enzyme systems and morphogenesis in cultures (Bonga, 1982).In an experiment made on rose shoots, replacing Fe-EDTA by Fe-EDDHA in Lepoivre and MS resulted in the development of green shoots for over 3 months. Addition of the light absorbing dye, fast yellow 9, to Lepoivre with Fe-EDTA also resulted in green shoots. (Van der Salm, 1994)

1.7.8 Auxin plus riboflavin

Riboflavin is also known as vitamin B2. It has been found to inhibit callus formation and may promote the growth and quality of the shoots (Drew and Smith, 1986). The oxidation of IAA *in vitro* may be promoted by plant pigments as riboflavin and also by light. (Taiz and Zeiger, 1991) This is a big advantage when using riboflavin and IAA in the rooting medium, since as

soon as the containers are moved into light, riboflavin and light will decompose the auxin and consequently the transfer into hormone-free medium is unnecessary.

1.7.9 Influence of in vitro formed roots on the ex vitro establishment

Thomas and Ravindra (1997) pruned or totally removed *in vitro* formed roots of micropropagated *Vitis vinifera* L. at planting and found that the *ex vitro* performance was unaffected by the practice with 97% and 90% establishment respectively in each treatment (92% for control plantlets). Both pruning and removal showed better adventitious root regeneration at the base compared with control plantlets with intact roots.

In an experiment by Gribaudo et al. (1995) to determine the faith of roots produced *in vitro*, the roots were stained with a dye before transplanting them and it was found that over 80% survived the *ex vitro* establishment.

In a study made by Welander and Huntrieser (1981) working with micropropagation of apple rootstock A2, neither shoot length nor root length influenced the survival percentage.

However, de Klerk (2000) found that the *ex vitro* performance and establishment of microcuttings of apple was correlated with the number and length of *in vitro* formed roots.

1.8 Objectives

This study aimed to optimise the methods for propagation of *Aristolochia manshuriensis* either by conventional cuttings or by optimising the multiplication and rooting media for micropropagation. The objective was to find an effective multiplication method that in a relative short time gave a high rate of rooted plants in good vigour to be able to meet the potential demands of the consumers.

2 Material and methods

Pretreatment of mother plants for cutting experiments ex vitro

The experiments were conducted with 32 mother plants, 17 originated from Balsgård, 5 from Alnarp and 10 from *in vitro* experiment. The plants from the *in vitro* experiment were the youngest ones with no woody parts. The others were older and had woody parts at the base. All were planted in pots of 16 cm in diameter and were grown for 45 days in the greenhouse.

Mother plants were grown for 52 days in a climate chamber at the Biotron in Alnarp, with 16 hours day length, at 22/18ºC day/night temperature and 70% air humidity. When cultivating the mother plants, they were divided into two groups. The first group (Gr.1) consisting of 9 plants from Balsgård, 2 from Alnarp and 5 from *in vitro*, were grown at 200 µmol m^{-2} s^{-1}. The second group (Gr.2) consisting of 8 plants from Balsgård, 3 from Alnarp and 5 from *in vitro* and were grown at 20 µmol m^{-2} s^{-1}.

The plants were given liquid nutrients (manufactured by Bayer AB, Lomma). The composition in percent of it's weight is; N 7.0%, P 2.2%, K 5.0%, S 0.04%, B 0.01%, Fe 0.02%, Cu 0.01%, Mn 0.01%, Mo 0.005%, Zn 0.005% and Mg 0.006%. The nutrition was given two to three times a week to the mother plants in a ratio of 4 ml liquid nutrition per liter water.

Totally 110 cuttings were taken from each group of plants, and it was recorded from which plant each cutting originated in an intent to see if the plant origin influenced the results. All cuttings were cut diagonally and then split.

Fig. 2.1: The explants are first cut in a sharp angle and then the cut area is split

Treatment of cuttings

In the experiments 220 cuttings were used. Half of them were from mother plants grown at 200 µmol m^{-2} s^{-1} and the other half at 20 µmol m^{-2} s^{-1}. They were dipped in IAA, IBA, glucose and IAA, glucose and IBA or untreated, as control cuttings. The concentrations used are given in table 2.1. The cuttings were dipped for 30 seconds. 20 cutting were used for each type of mother plant and treatment except for the auxins with glucose where only 15 cuttings each were used.

Figure 2.2: Mother plant etiolation tent with light intensity of 20 µmol m^{-2} s^{-1} with closed curtains

Table 2.1: Hormone dipping with IAA and IBA, with and without glucose

Light intensity	Without hormone	IAA 250 mg/l	IAA 500 mg/l	IBA 500 mg/l	IAA 500 mg/l and glucose	IBA 500 mg/l and glucose
200 µmol m^{-2} s^{-1}	20 cuttings	20 cuttings	20 cuttings	20 cuttings	15 cuttings	15 cuttings
20 µmol m^{-2} s^{-1}	20 cuttings	20 cuttings	20 cuttings	20 cuttings	15 cuttings	15 cuttings

Fig. 2.3: Cuttings taken from treated mother plants

Fig. 2.4: Tent used for *ex vitro* cuttings

After dipping, the cuttings were planted in small 6.5 x 6.5 cm containers in a mixture of 50% perlite and 50% peat (Weibulls kronmull with 10 volume percent haydite, lime and fertilizers added). Since *Aristolochia manshuriensis* often have quite big leaves, they were cut back to a size where less water is lost by transpiration. The containers were put in the greenhouse in a tent covered with a thick plastic sheet, with some openings to assure airflow.

Micropropagation of shoots for in vitro rooting

Micropropagated shoots used for rooting *in vitro* were produced in glass jars for an average of 28 days. The medium for shoot production contained Lepoivre macro nutrients, MS micronutrients, MS vitamins, 0.2 mg/l BAP, 30 g/l sucrose, 7.0 g/l agar, sterile water and the pH was adjusted to 5.5 before autoclaving for 20 min at 120º C.

The glass jars were placed in a climate chamber under coolwhite fluorescent tubes at 90-100 µmol m^{-2} s^{-1}.

Fig. 2.5: All work with the explants *in vitro* is done in a laminar flow bench. The picture shows when cuttings are transferred to jars containing nutrient medium with agar

Rooting in vitro

The rooting medium used consisted of 1/3 Lepoivre macro nutrients, MS micronutrients, MS vitamins, auxins, 40 g/l sucrose and 6.5 g/l agar. The pH was adjusted to 5.5 prior to autoclaving for 20 min at 120º C.

When the shoots were placed in rooting medium, they were kept in darkness for four days and then transferred to hormone free medium containing 1/2 Lepoivre macro nutrients, full MS micronutrients, full MS vitamins, 40 g/l sucrose, 6.5 g/l agar. The cultures were kept in a climate chamber with coolwhite fluorescent tubes at a light intensity of 40 µmol m^{-2} s^{-1}. The rooting experiments were conducted in square, plastic Magenta boxes of 325 ml, containing 60 ml rooting medium and five explants per container. All explants were cut in a sharp angle and then the cutting area was split in two.

Fig. 2.6: Plantlet under plastic cup

After examining the rooting of the plantlets, they were planted in small 6.5 x 6.5 cm containers in peat with 10 volume percent haydite (Weibulls kronmull with lime and fertilizers added). The containers were then placed in the greenhouse, each plantlet covered with a plastic cup that was leaning to assure airflow (see figure 2.6).

Reducing cytokinin content before rooting

The explants were grown on hormone free medium for 35 days. Before rooting some explants developed roots in the hormone free medium. These roots were removed before transferring them to the rooting medium containing IAA 20 mg/l, IBA 10 mg/l and NAA 5 mg/l.

Different types and concentrations of auxins

The auxins and levels tested were 10 and 20 mg/l IAA, 5 and 10 mg/l IBA and 2.5 and 10 mg/l NAA. Each hormone type and level was tested with 10 shoots.

Reducing macro nutrients to 1/3 and 1/5

In these experiments, the macro nutrients was reduced to 1/3 and 1/5 and combined with IAA, IBA and NAA at different levels (table 2.2). After four days in darkness the shoots were transferred to hormone free medium containing 1/2 Lepoivre macro nutrients, full MS micronutrients and full MS vitamins. The experiment was performed with 25 explants per auxin and concentration.

Table 2.2: IAA, IBA and NAA combined with 1/3 and 1/5 macro nutrients in the rooting medium

IAA 10 mg/l	IBA 5 mg/l	NAA 2.5 mg/l
IAA 15 mg/l	IBA 10 mg/l	NAA 5 mg/l
IAA 20 mg/l	IBA 15 mg/l	

Auxin in the rooting medium with and without AC

Activated charcoal was added to the hormone free rooting medium in order to lower the effects when a cutting is dipped in a high concentration of hormones and to remove undesirable phenols produced by the explants. The activated charcoal was added after adjusting the pH in the medium and then it was autoclaved for 20 min at 120º C.

The first experiment was performed with 10 g/l activated charcoal and different auxins according to table 2.3. The explants were placed in auxin containing medium in darkness for four days. The explants were then transferred to a hormone-free medium supplemented with 10 g/l of activated charcoal and moved into light. The different combinations were tested with 25 explants each.

Table 2.3: IAA, IBA and NAA in combination with activated charcoal

Activated charcoal	IAA 30mg/l	IBA10mg/l	NAA 2,5mg/l
10 g/l	X	X	X
5 g/l	X	X	X
2,5 g/l	X	X	X
None	X	X	X

The experiments with 10 g/l of activated charcoal failed to produce any roots though it was left longer than other experiments in the climate chamber. Therefore the same experiment was repeated with 5 g/l and 2.5 g/l activated charcoal.

The different combinations were tested with 25 explants each.

Auxin in the dipping solution with and without AC

The explants were dipped into the hormone solution for 15, 30 and 60 minutes. The hormone combinations were according to table 2.4.

Each dipping time and hormone was tested with 50 explants, 25 of them were put in hormone free medium with activated charcoal 10 g/l, and 25 of them in hormone free medium without activated charcoal. In total 300 explants were used.

Table 2.4: IAA and IBA with different dipping times, with and without activated charcoal

Activated charcoal 10 g/l	IAA 250 mg/l 15 min	IBA 250 mg/l 15 min	IAA 250 mg/l 30 min	IBA 250 mg/l 30 min	IAA 250 mg/l 60 min	IBA 250 mg/l 60 min
+	X	X	X	X	X	X
–	X	X	X	X	X	X

Altering the N-source

Glutamine was supplied as sole nitrogen source to the shoot production medium by altering the Lepoivre macronutrient basis solution. The Lepoivre macronutrient solution contained a total of 0.14 g N/l, with a nitrogen concentration corresponding to 0.37 g/l glutamine.

The explants were first grown in shoot production medium containing glutamine as the only nitrogen source. The shoots were then rooted on medium containing 1/3 Lepoivre macro nutrients and 20 mg/l IAA.

In an additional test the same quantity of glutamine as the only nitrogen source was supplied directly to the rooting medium.

Altering the Fe-source

Two different macronutrient solutions were made where Fe-EDDHA (Fe-ethylenediamine-di(*o*-hydroxyphenyl)acetic acid) was replacing Fe-Na-EDTA (Fe-ethylenediaminetetracetic acid) in the Lepoivre macro nutrients.

The explants were first grown in shoot production medium containing 40 and 80 mg/l Fe-Na-EDTA or Fe-EDDHA. The shoots were then rooted on medium containing 1/3 Lepoivre macro nutrients and 20 mg/l IAA.

The different combinations were tested with 25 explants each.

Auxin plus riboflavin

Riboflavin was supplied in two levels, 0.94 and 1.88 mg/l to the rooting medium containing 1/3 Lepoivre macro nutrients and 20 mg/l IAA. Each level was tested with 40 explants.

Riboflavin degrades IAA as soon as the explants are transferred into light and therefore it is not necessary to transfer the explants to hormone-free medium.

3 Results

3.1 Cutting experiments ex vitro

3.1.1 Distribution of cuttings

The different mother plants gave between 1 to 17 cuttings. The number of cuttings from mother plants with different origin differed significantly. Mother plants number 1–9 and 17–24 came from Balsgård. Number 10-11 and 25-27 came from Alnarp and number 12–16 and 28–32 came from *in vitro*. The mean number of cuttings was 9.1 from Balsgård, 6.2 from Alnarp and 3.4 from *in vitro* mother plants.

In total 220 equal cuttings in good vigor was taken from the mother plants including 110 cuttings taken from mother plants cultivated in Gr.1 and 110 cuttings from mother plants in Gr.2.

The mother plants from Gr.2 grown in the climate chamber for 52 days in the shaded area seemed more vigorous, greener and had a healthier appearance than Gr.1.

3.1.2 Rooting ex vitro

The rooting process for *A. manshuriensis* is very slow and good rooting was in some cases not obtained until after 34 weeks. The cuttings have a tendency to stay green during a long time, even without callus or root formation. In some cases the shoot can produce new leaves without having produced roots. Figure 3.1 shows a vigorous well rooted cutting with a new leaf. Figure 3.2 shows a green cutting without roots yet with a new leaf which shows signs of insufficient nutrient supply to the right of the original one and figure 3.3 shows a cutting without roots or callus but still green. The pictures were taken after 22 weeks.

Fig 3.1: Well rooted cutting with a new green leaf on the left side of the original one after 22 weeks

Fig 3.2: Cutting with a new leaf on the right side of the original one but still without roots after 22 weeks

Fig 3.3: Cutting without roots or callus formation but still green after 22 weeks

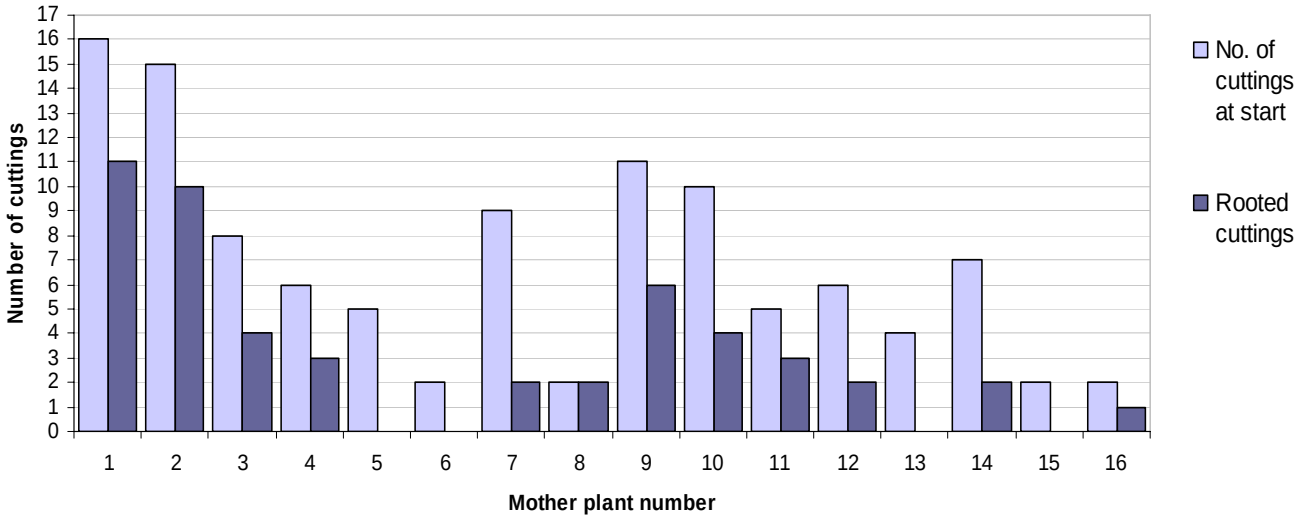

Fig. 3.4: Number of cuttings originally taken from the different mother plants and number of rooted cuttings after 34 weeks. Number 1-16 cultivated at 200 µmol m^{-2} s^{-1} (Gr.1)

Mother plants 1-9 from Balsgård, 10-11 from Alnarp and 12-16 from *in vitro*

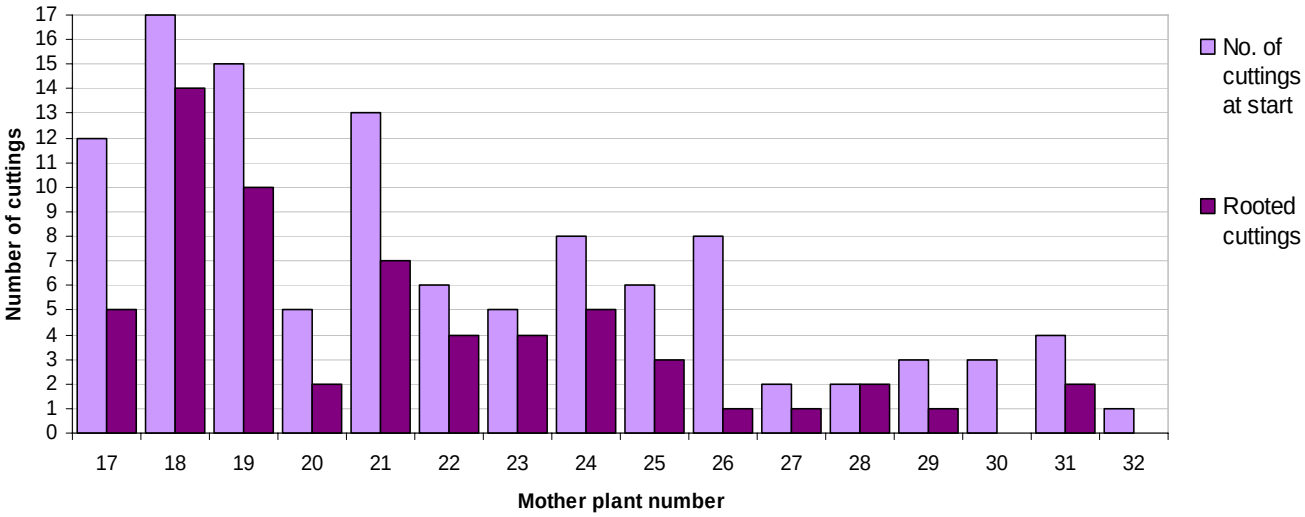

Fig. 3.5: Number of cuttings originally taken from the different mother plants and number of rooted cuttings after 34 weeks. Number 17-32 cultivated at 20 µmol m^{-2} s^{-1} (Gr.2)

Mother plants 17-24 from Balsgård, 25-27 from Alnarp and 28-32 from *in vitro*

Fig. 3.4 shows mother plants 1-16 cultivated at 200 µmol m^{-2} s^{-1} (Gr.1) and Fig. 3.5 shows mother plant 17-32 cultivated at 20 µmol m^{-2} s^{-1} (Gr.2) and the number of cuttings taken from each mother plant along with the number of rooted cuttings obtained after 34 weeks. Generally Gr.2 gave a higher percent of rooted cuttings (55%) than Gr.1 (45%). The mother plants from Balsgård gave 155 cuttings and 57% of them rooted. The ones from Alnarp gave 31 cuttings and 39 % rooted. The *in vitro* ones were 34 and 29% rooted.

The highest rooting percentage was obtained from mother plant number 8 from Alnarp and 28 from *in vitro* (100%). The highest number of rooted cuttings (14) came from the mother plant giving the highest number of cuttings (17) with a rooting percentage of 82%.

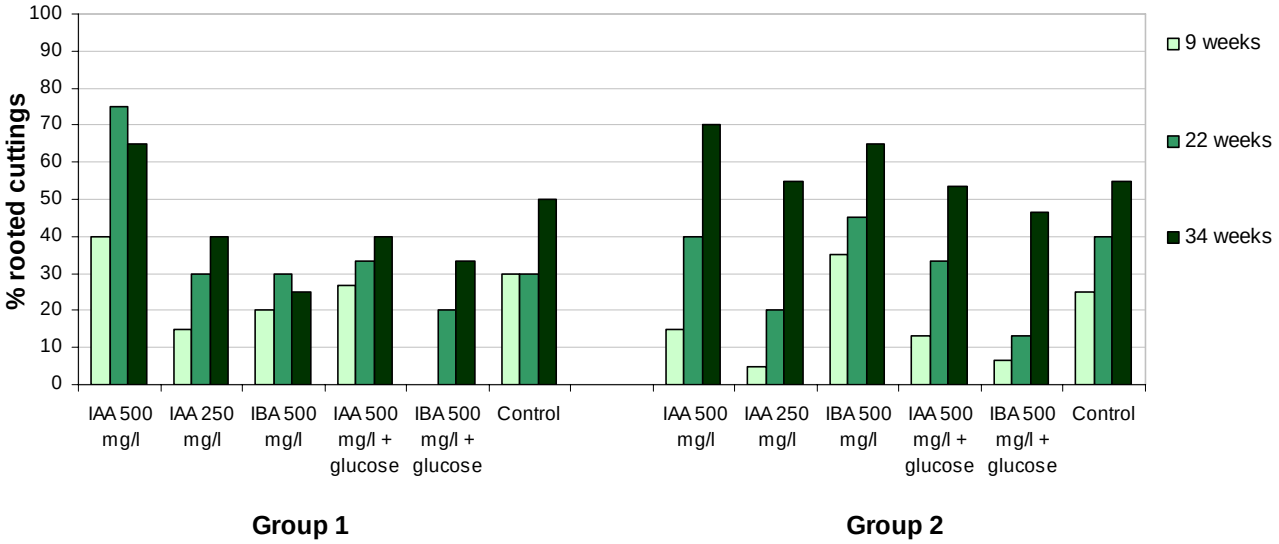

Fig. 3.6: Percent rooted cuttings treated with IAA and IBA in different concentrations, with and without sugar, and untreated controls of mother plants from Gr.1 and Gr.2 at 9, 22 and 34 weeks

Figure 3.6 shows that good rooting was not obtained until after 34 weeks except for the cuttings from Gr.1 obtained with IAA 500 mg/l which resulted in optimum rooting after 22 weeks. The untreated control cuttings have unexpectedly high rooting percent in both groups. The results also show that some rooted cuttings died during the treatment with both IAA and IBA 500 mg/l from Gr.1. After 34 weeks the highest rooting percentage was obtained with IAA 500 mg/l in both Gr.1 and Gr.2 (up to 70%). The overall rooting recorded was higher for Gr.2 cuttings. The rooting percent was not improved by combining auxin with sugar. All cuttings were either rooted or dead at 34 weeks.

The figure 3.7 shows the mean value for the vigor of the cuttings. They were rated from 0-3.

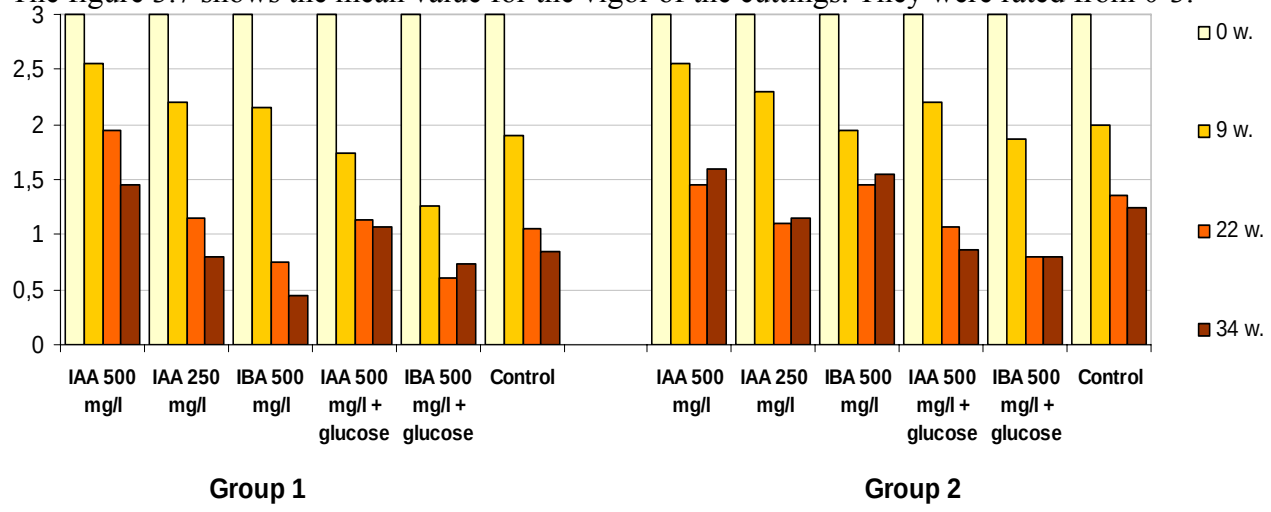

Fig. 3.7: Mean values for vigor of the cuttings from Gr.1 and Gr.2. They were rated from 0-3 where 0 = dead, 1 = alive but not well growing, 2 = a smaller healthy plant and 3 = healthy growing plant

From the beginning, all cuttings were rated 3. The most vigorous and healthy plants after 34 weeks were found in Gr.2.

3.1.3 Statistical analysis

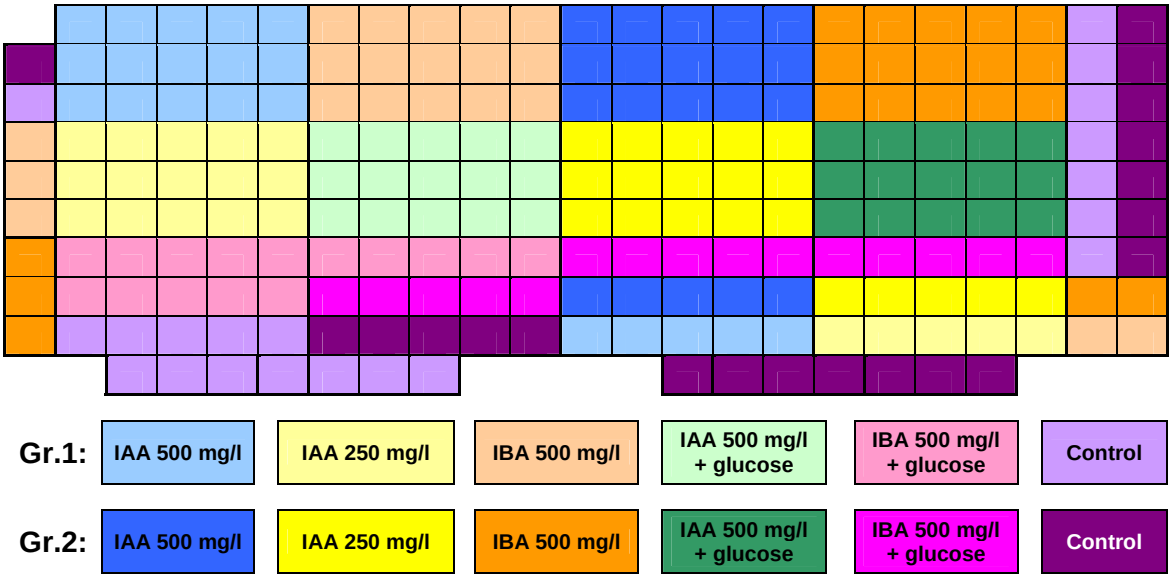

Fig. 3.8: The position of the cuttings as they were placed on the bench in the greenhouse. The treatments are represented by a grey scale

Figure 3.8 shows 220 squares each symbolizing one cutting. The squares in the figure are distributed as the cuttings were distributed on the bench in the greenhouse. The colours represent the different treatments. Cuttings from Gr.1 are represented by a brighter grey scale than Gr.2.

Gr.1 is located to the left in the figure and almost all cuttings from Gr.2 on the right side. The few exceptions from Gr.2 on the left side are 6 control cuttings, 3 IBA 500 mg/l and 5 IBA 500 mg/l + glucose cuttings. The ones from Gr.1 on the right side are 7 control cuttings, 5 IAA 250 mg/l and 2 IBA 500 mg/l cuttings.

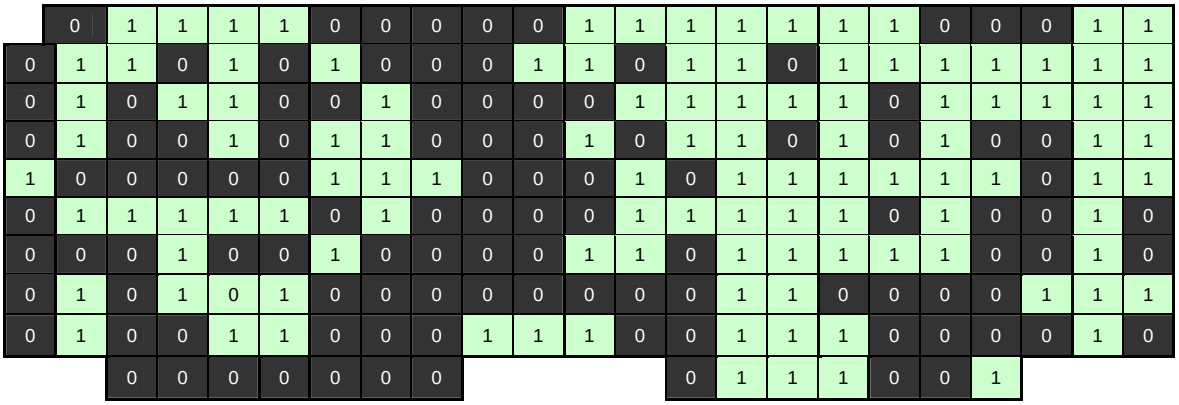

Fig. 3.9: The position of the cuttings and their survival ratios where 0 stands for dead and 1 represent alive, after 34 weeks *ex vitro* in the greenhouse

According to figure 3.9, the dead cuttings are to be found in clusters. The pattern of distribution differs significantly from a random pattern.

There was a higher number of rooted cuttings on the right side, which with few exceptions, are cuttings from Gr.2.

There is no relation between pattern of distribution and survival within the different treatments.

3.2 Rooting in vitro

For shoot production *in vitro*, jars of 300, 325 and 350 ml was used. The shoots grew better and had a healthy appearance for a longer time in the biggest one. A notable difference was that the lids didn't close very tight on this type of container.

In some cases shoots tends to wilt and die back before starting over from the base as seen in figure 3.10. The well-rooted plants grew really fast, taking away a lot of light from the smaller ones (figure 3.11). The cups were sometimes tipped over by the growing plant, but frequently the cups had to be removed by hand (figure 3.12).

The rooting results don't include rooted explants that did not survive and don't appear in the rooting statistics.

Fig. 3.10: Shoot that died back before developing roots and a new leaf

Fig. 3.11: Micropropagated plants growing in the greenhouse

Fig. 3.12: Plant growing inside plastic cup without tipping it over

3.2.1 Reducing cytokinin content before rooting

Table 3.1: Percent rooted explants in media with reduced cytokinin level prior to rooting. In the rooting media three different types of auxins at one level was used. Results after 3 weeks *in vitro*. N= 25

Treatment	IAA 20 mg/l	IBA 10 mg/l	NAA 5 mg/l
Rooting	16%	12%	8%

After 35 days in hormone free medium, 20% of the explants rooted. These roots were removed and the shoots were all cut the same way before transferring them to rooting medium containing different auxins. According to table 3.1, the obtained rooting percentages for IAA were 16%, IBA 12% and NAA 8% after 3 weeks *in vitro*. There were no dead explants in this experiment. The shoots from this experiment were not followed *ex vitro*.

3.2.2 Different types and concentrations of auxins

Table 3.2: Percent rooted explants over time after 5 weeks *in vitro* and 9 and 12 weeks *ex vitro*, on different types and concentrations of auxins. N= 10

Treatment	IAA 10 mg/l	IAA 20 mg/l	IBA 5 mg/l	IBA 10 mg/l	NAA 2.5 mg/l	NAA 10 mg/l
***In vitro* after 5 weeks**	20%	40%	20%	40%	30%	10%
***Ex vitro* after 9 weeks**	20%	20%	20%	40%	20%	20%
***Ex vitro* after 12 weeks**	10%	20%	70%	60%	0%	30%

Table 3.2 shows the rooting results over time with different types and concentrations of auxins. Both rooted and unrooted *in vitro* shoots were planted and followed *ex vitro*. After 12 weeks *ex vitro* the highest rooting percentage (70%) was obtained for IBA 5 mg/l. After 5 weeks *in vitro* the highest rooting percentage (40%) was obtained with IAA 20 mg/l and IBA 10 mg/l. With NAA 2.5 mg/l, 30% was rooted *in vitro* after 5 weeks, but all the rooted plantlets died after 12 weeks *ex vitro*. All cuttings that didn't root were dead after 12 weeks. Since this experiment was made with a small number of explants, there were no statistically significant differences.

3.2.3 Reducing macro nutrients to 1/3 and 1/5

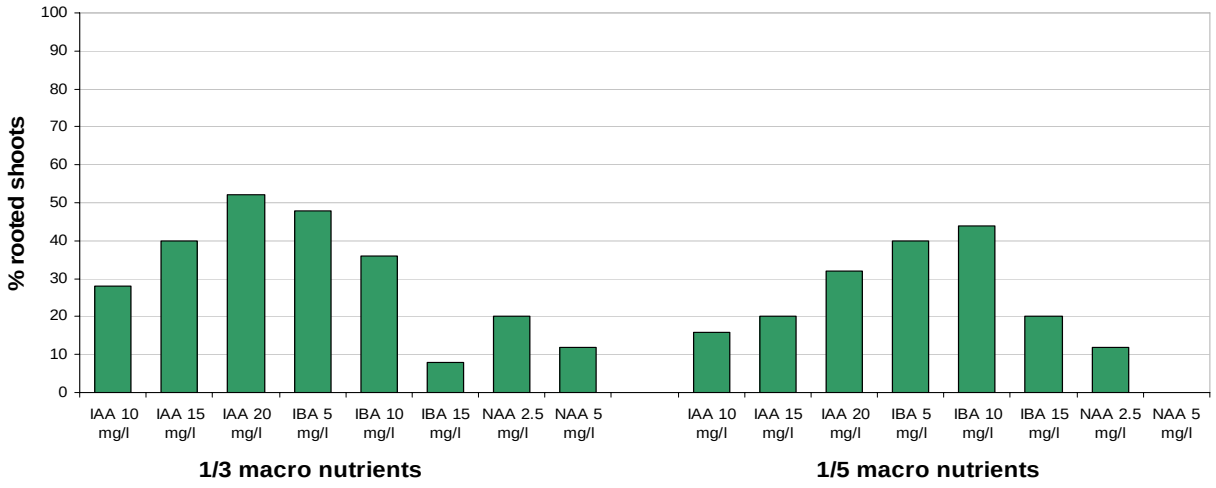

Fig. 3.13: Percent rooted shoots after 3 weeks *in vitro* with two different levels of macro nutrients and different types and concentrations of auxins. N= 25

Figure 3.13 shows that the best rooting overall was obtained with the 1/3 macro nutrients. The optimum rooting was obtained with IAA 20 mg/l (52%) and IBA 5 mg/l (48%) in the 1/3 group. The highest rooting percent for the 1/5 group was with IBA 10 mg/l (44%). For the 1/3 macro nutrients group some dead shoots were found within the IAA and NAA treated shoots. All explants survived rooted or not from 1/5 macro nutrients. The shoots from this experiment were not followed *ex vitro*.

There was a difference in vigor between the two groups rooted with 1/3 and 1/5 macro nutrients. The 1/5 macro nutrients group was greener and healthier than the other group. In general the treatments with higher rooting percent had more yellow shoots and the treatments with lower rooting percent had greener and more vigorous shoots. IBA had a higher number of yellow shoots than IAA in both groups. IBA 10 and NAA 2.5 mg/l in the 1/5 macro nutrient group had the highest number of roots per rooted shoot. (Data not shown).

Figure 3.14 shows that although the IBA 10 mg/l treatment had the highest rooting percent of all in the 1/5 macro nutrient group the shoots were not in good vigor.

However shoots from NAA 2.5 mg/l treatment in the 1/5 macro nutrients group were in good vigor although this treatment had the lowest rooting percent of all in the 1/5 macro nutrient group (Fig. 3.15).

Fig 3.14: Shoots treated with IBA 10 mg/l from the 1/5 macro nutrient group showing prominent callus and root formation

Fig 3.15: Shoots treated with NAA 2.5 mg/l from the 1/5 macro nutrient group with lots of callus and lots of roots

In this experiment callus formation was recorded according to figure 3.16 (A, B and C). The scale ranged from 0-2, where 0 = no callus, 1 = some callus and 2 = lots of callus.

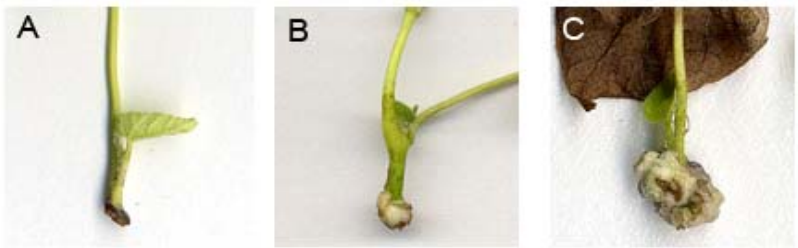

Fig. 3.16 A, B and C: The callus was recorded in a scale from 0-2. A shows "callus 0", B shows "callus 1" and C shows "callus 2"

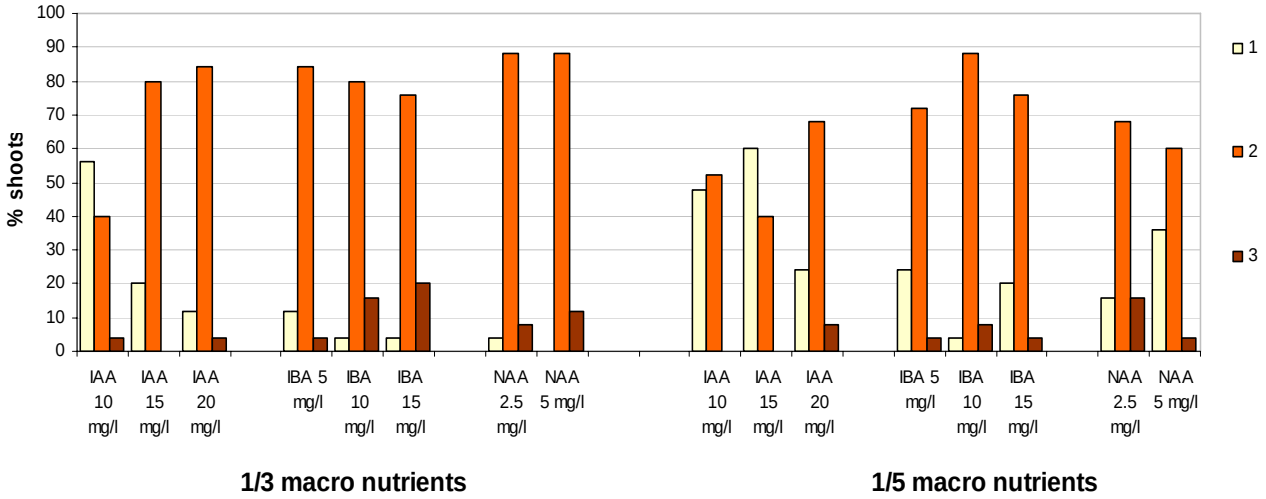

Fig. 3.17: Percent shoots with different callus formation from the experiment with 1/3 and 1/5 macro nutrients after 3 weeks *in vitro*. 0 = no callus, 1 = some callus or 2 = lots of callus

According to figure 3.17, the callus formation is less for IAA then for IBA and NAA. There were more callus formation in general for the explants treated with 1/3 macro nutrients. For the 1/3 macro nutrients group, the callus formation were notable high for IBA 10, IBA 15 mg/l and for both levels of NAA and high numbers of yellow shoots were recorded for these treatments and for IAA 20 mg/l. The least callus formation was recorded for IAA 10 and IAA 15 mg/l in the 1/5 macro nutrient group.

IAA had low levels of callus in both nutrient groups, but higher rooting percent in the 1/3 macro nutrient group. For the IAA treated shoots the tendency was that the more callus formation the higher the rooting percentage. Both NAA levels in both nutrient groups have a lot of callus, but low rooting.

3.2.4 Auxin in the rooting medium with and without AC

Table 3.3: Percent rooted and survived shoots after 8 weeks *in vitro* and 12 weeks *ex vitro* treated with different auxins and transferred to hormone free medium with 0 g/l or 10 g/l activated charcoal, N= 25

	8 weeks *in vitro*						12 weeks *ex vitro*					
Hormones	**IAA 30 mg/l**		**IBA 10 mg/l**		**NAA 2.5 mg/l**		**IAA 30 mg/l**		**IBA 10 mg/l**		**NAA 2.5 mg/l**	
10 g/l AC	**–**	**+**	**–**	**+**	**–**	**+**	**–**	**+**	**–**	**+**	**–**	**+**
% rooted	28	0	16	0	16	4	8	28	12	52	0	0
% survived	72	100	76	100	80	96	0	0	0	0	0	0

Table 3.3 shows percent rooted explants treated with IAA, IBA and NAA, with or without 10 g/l AC recorded after 8 weeks *in vitro* and 12 weeks *ex vitro*.

After 8 weeks the highest rooting percentage was obtained with IAA 30 mg/l without AC. However after 12 weeks some rooted explants have died and therefore a lower rooting per-

centage was recorded. The same thing was noted for IBA and NAA without AC. After 8 weeks with AC there were no dead explants at all.

All surviving explants after 12 weeks were rooted. After 12 weeks, IBA 10 mg/l gave significantly higher rooting percentage than the rest of the treatments both with and without AC. After 12 weeks the IAA treated shoots without AC were rooted but yellow and all NAA treated shoots were dead (see table 3.3).
The explants in figure 3.18 had lots of callus and were yellowish with dark areas meanwhile the ones treated with activated charcoal were greener and more vigorous (Fig.3.19).

Fig 3.18: Shoots from IBA 10 mg/l without activated charcoal after 8 weeks

Fig 3.19: Shoots from IBA 10 mg/l and with 10 g/l activated charcoal after 8 weeks *in vitro*

Table 3.4: Percent rooted shoots treated with different auxins and transferred to hormone free medium with 0 g/l, 2.5 g/l and 5 g/l activated charcoal. Recorded after 9 weeks *in vitro* N= 25

Treatment	IAA 30 mg/l	IBA 10 mg/l	NAA 2.5 mg/l
0 g/l AC	24%	8%	20%
2.5 g/l AC	0%	0%	0%
5 g/l AC	0%	0%	0%

Table 3.4 shows percent rooted explants treated with different auxins and different levels of AC recorded after 9 weeks *in vitro*. The highest rooting percentage was obtained with IAA 30 mg/l without AC. IAA with 5 g/l charcoal gave no dead and no rooted explants. The shoots from this experiment were not followed *ex vitro*.
The explants in figure 3.20 had lots of callus or were yellow with a dark stem meanwhile the ones treated with activated charcoal were greener and more vigorous (Fig.3.21).

Fig 3.20 Shoots from NAA 2.5 mg/l without activated charcoal after 9 weeks *in vitro*

Fig 3.21 Shoots from NAA 2.5 mg/l with 5 g/l activated charcoal after 9 weeks *in vitro*

3.2.5 Auxin in the dipping solution with and without AC

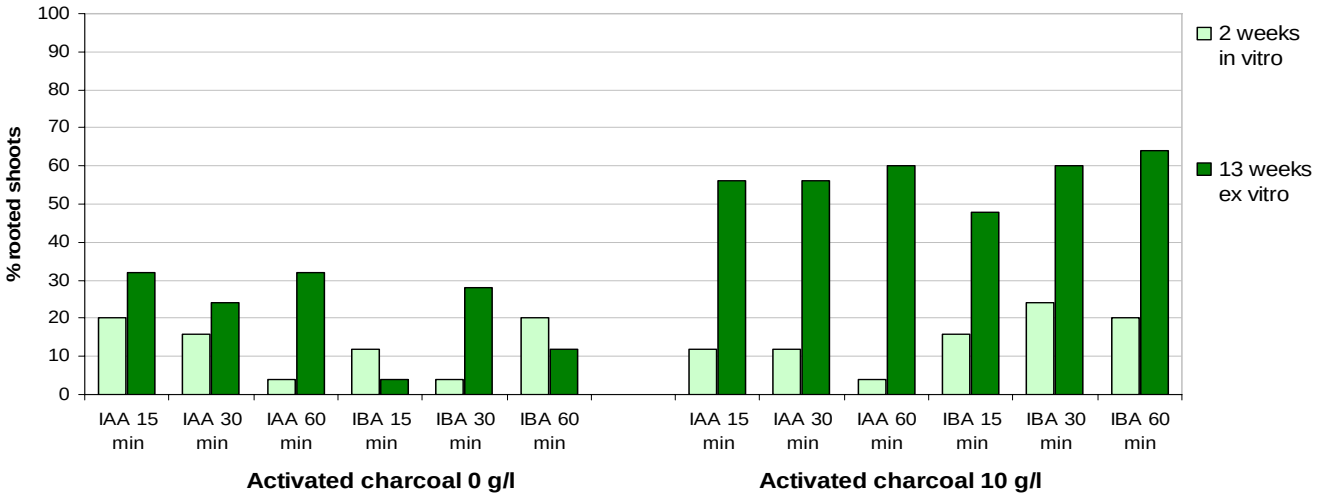

Fig. 3.22: Percent rooted explants after dipping in IAA or IBA 250 mg/l during three different times, and then transferred to hormone free medium *in vitro* containing 0 g/l or 10 g/l activated charcoal. Recorded after 2 weeks *in vitro* and 13 weeks *ex vitro*. N= 25

The cuttings were dipped *in vitro* and transferred to hormone free medium with or without AC. In general the treatments without AC had better rooting after 2 weeks *in vitro* and the treatments with 10 g/l AC had better rooting after 13 weeks *ex vitro*. Figure 3.22 shows that the best rooting percent was obtained with IBA 60 min (64%), with AC after 13 weeks. The best rooting percent after 2 weeks (24%) was obtained from IBA 30 min with AC. The survival percent were generally good. Dead explants were only found for IBA 60 min, with AC (4%) and without (8%). After 13 weeks all plants in both groups were either rooted or dead.

IBA 15 min gave a significantly lower overall rooting than the rest of the treatments. The group with activated charcoal had significantly higher rooting after 13 weeks.

The shoots in activated charcoal stayed more vigorous and they survived without roots for a longer time.

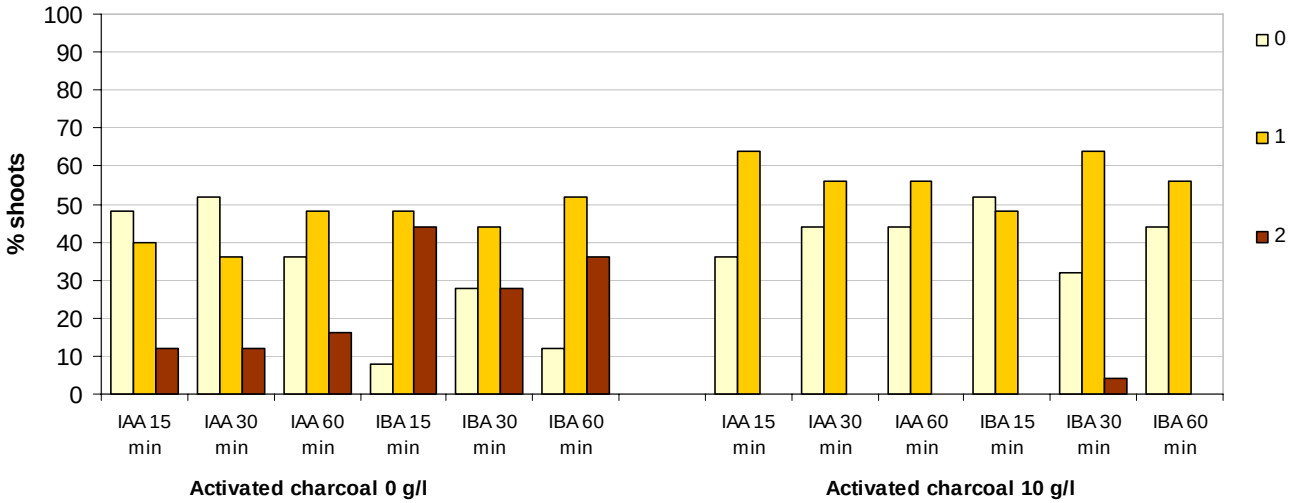

Fig. 3.23 Percent shoots with different callus formation after dipping in IAA and IBA 250 mg/l, and then transferred to hormone free medium containing 0 g/l or 10 g/l AC. Recorded after 2 weeks *in vitro*. 0 = no callus, 1 = some callus or 2 = lots of callus. N= 25

Figure 3.23 shows the amount of callus produced after 2 weeks on shoots after being exposed to auxins at different times and thereafter transferred to hormone free medium with or without AC. There was a significant difference in callus formation with and without AC after 2 weeks. The highest level of callus had IBA 15 min without AC which also had the lowest overall rooting.

3.2.6 Altering the N-source

Table 3.5: Glutamine as the only N-source in regeneration medium. N= 45

Glutamine	Regeneration
Surviving	11%
Dead	89%

Table 3.6: Glutamine as the only N-source in rooting medium. N= 45

Glutamine	Rooting
Rooted	20%
Dead	11%

According to table 3.5 using glutamine as only N-source shows poor results after 8 weeks *in vitro* in regeneration medium and the surviving shoots were too few to conduct a rooting experiment. Table 3.6 shows the explants cultivated in rooting medium containing glutamine after 8 weeks *in vitro*. The shoots from this experiment were not followed *ex vitro*.

3.2.7 Altering the Fe-source

Table 3.7: Percent rooted explants after 12 weeks *in vitro*, from shoots cultivated with different types and levels of iron supplied to the shoot production medium and rooted with IAA 20 mg/l. N= 25

Treatment	EDTA	EDDHA 40 mg/l	EDDHA 80 mg/l
Rooting	12%	16%	16%

Table 3.7 shows the rooting percent with iron as EDTA or replacing it with EDDHA 40 or 80 mg/l. The best results were obtained with EDDHA. EDTA had 20% dead explants, EDDHA 40 12% and EDDHA 80 mg/l had 24%. The shoots from this experiment were not followed *ex vitro*.

3.2.8 Auxin plus riboflavin

Table 3.8: Percent rooted explants treated with two different levels of riboflavin together with IAA 20 mg/l, *in vitro* after 12 weeks. N= 40

Treatment	Riboflavin 0.94 mg/l + IAA 20 mg/l	Riboflavin 1.88 mg/l + IAA 20 mg/l
Rooting	12.5%	20%

As seen in table 3.8, the optimum rooting capacity was for the 1.88 mg/l treated shoots. Both levels of riboflavin had 25% dead shoots. The shoots from this experiment were not followed *ex vitro*.

4 Discussion

4.1 Rooting ex vitro with cuttings from light treated mother plants

The results presented here indicate that the optimum conditions for rooting were not found since the type or level of hormones or type and level of additives did not improve the rooting percent significantly compared to the untreated ones.

The etiolated mother plants in this experiment gave a higher number of rooted cuttings. Improved rooting by etiolation has also been stated by Hartmann et al. (2002). It is further supported by studies with top cuttings from etiolated mother plants of *Scaevola aemula* (Welander, 1992) and the same results were obtained with node cuttings of *Populus tremula* (Welander, unpublished). Our results showed that the older mother plants of *Aristolochia manshuriensis* rooted better. This is probably species dependent since Wang and Andersen (1989) found that cuttings from older stock plants of *Hibiscus* rooted less and even after IBA treatment. Protacio et al. (2001) found that older nodal cuttings of *Aglaonemas* 'Silver Queen' and 'Emerald Beauty' rooted better than younger stem cuttings due to the presence of preformed root initials. However it is unlikely that this is the case for *Aristolochia manshuriensis* since rooting of this species is a very slow process.

In our results, only etiolated cuttings showed enhanced rooting when dipped 30 seconds in IBA 500 ppm compared to untreated ones. However, Protacio et al. (2001) showed that for *Aglaonemas*, etiolated cuttings as well as light grown ones, significantly produced more roots than untreated ones when dipped 15 minutes in 50 ppm IBA.

According to our results, IAA gave higher rooting percentage than IBA and NAA. However JongSuk et al. (2002) obtained the highest rooting percentage for *Styrax japonica* for cuttings treated with IBA. In softwood cuttings of *Styrax japonica* treated with IBA and IAA, callus formation and rooting occurred at the same time wheras in our experiments for *Aristolochia*, IAA treated cuttings rooted earlier than IBA or NAA treated ones.

The dipping in auxin solution for 30 sec did not give the desired results and therefore a prolonged exposure time might be better. Chauhan, et al. (1994) found that dipping in IBA and NAA for 24 h compared with a quick dip gave better results for cuttings of three different species (*Woodfordia floribunda, Coriaria nepalensis* and *Debregeasia hypoleuca*) regarding sprouting, callusing and rooting.

There were some problems with survival *ex vitro* in the greenhouse. From beginning the mother plants were infested by the twospotted spider mite, *Tetranychus urticae*. The plants were treated with insecticides but the attack was severe and afterwards the shoots and cuttings in the greenhouse were infested. Later all affected leaves were removed from the plantlets, thereby keeping the spider mite population to a minimum. The plants were also attacked by fungus gnats, *Sciára thomae* and shore flies, *Scatella stagnalis*. Too little drainage resulted in rapid growth and reproduction of these species. They were biologically treated with the insect *Hypoaspis miles* and also reduced by less irrigation. Later the plants were also attacked by whiteflies, *Trialeurodes vaporariorum*, and small black snails (*Limax* or *Arion* species) that ate holes in the foliage and even whole leafs, leaving only a part of the middle vein.

The survival of cuttings in the greenhouse was likely to also have been dependent on the position of the cuttings on the bench. During the summer, part of the bench in the greenhouse was flooded by the automatic irrigation but it left the corners dry. Rooted cuttings of *Aristolochia manshuriensis* appeared to tolerate draught better than great quantities of water and thus in-

fluenced the survival. Dead cuttings were found in clusters and the pattern of distribution differed significantly from a random pattern.

4.2 Rooting in vitro

4.2.1 Reducing cytokinin content before rooting

The shoots in hormone free media started to root. These results are in accordance to Humphries (1960) showing that cytokinins in the medium can inhibit or delay root formation and prevent root growth. However, after removal of the roots, the remaining shoots treated with auxins had a low rooting percent. In further studies it can be beneficial transferring the explants to cytokinin free medium also containing gibberellic acid before rooting the shoots in hormone free medium as proposed by Snir and Erez (1980).

4.2.2 Different types and concentrations of auxins

The highest level of NAA resulted in a higher rooting percent after 12 weeks *ex vitro* than after 5 weeks *in vitro*. This might be explained by the fact that the auxin level initially was too high for root elongation but appropriate to initiate more roots when the inhibitory level decreased. The lowest concentration of NAA gave a higher rooting percent after 5 weeks than after 12 weeks which means that rooted plantlets have died. According to Hartmann et al. (2002), auxin stimulates root initiation but inhibit root elongation and therefore NAA at high concentration can influence rooting negatively.

The rooting percent for shoots treated with IAA decreased after 12 weeks compared to 5 weeks because rooted shoots have died during the experiment. However for IBA treated shoots, the number of rooted shoots increased over time. It can be that IAA concentration was too low at the beginning. According to Nissen and Sutter (1990) IAA is rapidly degraded by light, even by cool-white fluorescent tubes and this might explain the low rooting after some time. The gradually increased rooting for IBA might be due to the fact that IBA is more stable than IAA. In future investigations it would be interesting to try a combination of equal parts of IBA and NAA since they have shown to be more effective for root formation and give a higher amount of roots when used on a number of different species than either component alone (Hartmann et al. 2002).

4.2.3 Reducing macro nutrients to 1/3 and 1/5

There were no signs that lowering the nitrogen level for *Aristolochia manshuriensis* in the rooting medium could increase rooting as Hartmann et al. (2002) have established. On the contrary the rooting increased with the higher level of macro nutrients. This could be a sign that this species is especially nutrient demanding. It is also possible that another macro nutrient besides nitrogen is the limiting factor. The plantlets from the 1/5 macro nutrient treatment seemed greener and did not die back in the same extent. It may be because they didn't put the energy in developing roots.

The callus formation was higher for IBA and NAA, than for IAA. This could be explained by the fact that light deteriorates IAA, meanwhile the IBA and NAA stays longer in the tissues (Hartmann et al., 2002). In general there is less callus formation for the group treated with 1/5 macro nutrients which also had a lower rooting percentage.

4.2.4 Auxin in the rooting medium with and without AC

Addition of 2.5, 5 and 10 g/l activated charcoal in the hormone free media did not improve rooting. The low rooting percentage obtained can be explained by the facts that activated charcoal adsorbs unwanted exudates but it will also remove essential compounds from the

medium such as phenolics, auxins, cytokinins, ethylene, vitamins, iron chelates but not sucrose (Bonga and von Aderkas, 1992; Pierik, 1997). For example activated charcoal in a concentration of 0.1 – 5% reduced an initial concentration of 10 μM IAA and IBA in liquid medium by 97% (Nissen and Sutter, 1990).

4.2.5 Auxin in the dipping solution with and without AC

However when shoots from *Aristolochia* were dipped in auxins and then transferred to solid media with activated charcoal, a high rooting percent was obtained after 13 weeks. According to George (1993), activated charcoal is beneficial for root development. The activated charcoal also influenced callus formation. After 2 weeks there was statistically more callus on shoots in medium with activated charcoal. Shoots, grown in medium without activated charcoal, had more callus when dipped in IBA compared to IAA which is supported by Hartmann et al. (2002) who states that IBA stays longer in the tissues than IAA. In general, treatments with lots of callus resulted in lower rooting percent than treatments with low callus formation. The concentration of hormone compared with the content of activated charcoal, needs to be balanced since too much activated charcoal or too low concentration of auxin slow down the rooting process.

4.2.6 Altering the N-source

Adding glutamine to the shoot production medium did not improve shoot production. According to George (1993) plants converts nitrogen into amino acids before assimilating them and therefore the cutting could save energy if the nitrogen was provided as amino acids from the beginning. Wetherell and Dougall (1976) successfully used glutamine as sole nitrogen source when cultivating tissues from wild carrots.

Shoots on glutamine containing media were not as vigorous as the shoots on the original one. This could be a sign of vitrification, also called hyperhydration. According to Pierik (1997) it is mainly a result of too high humidity in the growth container or too low agar concentration in the solid media. Vitrification can be reduced, as for cultures of *Prunus dulcis* when replacing sucrose by fructose in the medium (Rugini et al., 1986) and vitrified shoots could be reverted to normal ones when transferred to medium with ½ total nitrogen according to McLaughlin and Karnosky (1989).

4.2.7 Altering the Fe-source

The results obtained here show a somewhat higher rooting percent for both levels of Fe-EDDHA than for Fe-EDTA. In studies made on rose shoots by Van der Salm (1994) replacing Fe-EDTA by Fe-EDDHA in Lepoivre and MS resulted in the development of green shoots for over 3 months.

4.2.8 Auxin plus riboflavin

The riboflavin experiment was done with the same quality of mother plants *in vitro* as the experiment with glutamine. They did not look as vigorous as for the other experiments. This might explain our poor results on rooting with riboflavin. In the literature it is shown that riboflavin can either inhibit or improve rooting. In a study on *in vitro* rooting of the almond × peach hybrid clone GF 667, a low percentage of rooted shoots were obtained when adding riboflavin to the rooting media together with IBA. Some of the shoots developed symptoms of chlorosis and the highest concentration of riboflavin totally inhibited rooting. It could be explained by the concentrations of Fe and Zn which increased and reached toxic levels. (Antonopoulou, et al., 2005) This is contradicted to the results obtained by Drew and

Smith (1986) working with *Carica papaya* L. which states that riboflavin promoted the growth and quality of the micropropagated shoots.

4.3 Conclusions

The best rooting results within all experiments *in vitro* were equal to the best results from the experiments with cuttings from light treated mother plants *ex vitro*. The difference between the two groups was in efficiency. The highest rooting percent for cuttings from light treated mother plants were obtained after 34 weeks when all cuttings were either rooted or dead. Within the micropropagated *in vitro* experiments, similar rooting percent was obtained already after 12 weeks. Although the optimal conditions for *in vitro* rooting have not been obtained, the micropropagation technique is superior to production by *ex vitro* cuttings due to the time reduction and also since a larger quantity of shoots can be obtained by micropropagation.

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