AN ABSTRACT OF THE THESIS OF

Giovanni Iapichino for the degree of Master of Science in Horticulture presented on April 24, 1990.

Title: Micropropagation and In Vitro Plant Regeneration of Rhododendrons

Abstract approved: ______________________________

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The majority of tissue culture studies on *Rhododendron* have focused on micropropagation by the induction of axillary shoot growth. This type of shoot multiplication has high genetic stability since somaclonal variation is minimized. There has been no report concerning micropropagation of *Vireya* rhododendrons. *In vitro* propagation by adventitious shoot regeneration from somatic tissue offers a more efficient method for massive plant multiplication since a greater number of plants can be produced from a single explant. Furthermore, the ability to regenerate whole plants from somatic tissue is a fundamental prerequisite for applying modern gene transfer techniques to the genetic improvement of *Rhododendron*.

The objectives of this study were: 1) to develop an efficient micropropagation system for the hybrid *R. laetum*

x *aurigeranum*; and 2) to explore the feasibility of plant regeneration from somatic tissues of *R. laetum* x *aurigeranum* and commercial cultivars of *Rhododendron*.

To realize objective I, shoot tips taken from greenhouse grown plants were cultured on Anderson's medium supplemented with different combinations of IAA and 2iP concentrations. Optimal axillary shoot proliferation was observed after three months of culture in medium supplemented with 15 mg/l 2iP. After 6 months of culture, brown callus developed at the cut surfaces of the original explant. This callus produced a large number of adventitious shoots. Clumps of adventitious shoots that were divided and subcultured lead to greater shoot proliferation.

In this study three types of shoot multiplication were observed in *R. laetum* x *aurigeranum*: 1) multiple shoot formation from shoot tip culture (axillary shoots); 2) adventitious shoot formation from callus produced at the cut surfaces of the primary shoot tip explant; and 3) adventitious shoot regeneration from the base of individual shoots. We estimated a 137, 1600 and 7.8 fold increase in shoot number over six months for type 1, 2 and 3 shoot multiplication types respectively. Of these shoot forms, the second type was the most efficient for micropropagation of *R. laetum* x *aurigeranum*. *In vitro* rooting was accomplished by lowering the Anderson's medium to 1/4 ionic strength and by adding IAA (5 mg/l). Plants adapted well to

the soil environment and did not show any visible morphological abnormality.

Plant regeneration from different somatic tissues of R. laetum x aurigeranum was tested. The highest percent of regeneration was obtained from leaf strips and leaf sections cultured on Anderson's medium supplemented with 2 mg/l IAA and 20 mg/l 2iP. The leaf strip explants required a long intermediary callus phase (6 months) before organogenesis occurred. When whole leaves devoid of petiole end were used, we found that shoot primordia differentiation could be accomplished in 2 months.

Leaf strip explants of 10 commercial Rhododendron cultivars were cultured on Anderson's medium supplemented with various combinations of IAA and 2iP concentrations. Genotypic differences were observed in in vitro shoot regeneration. Among the 10 cultivars tested only three ('Ivory Coast', 'Joe Paterno' and 'Lodestar') showed reproducible plant regeneration response. Ivory Coast gave the best results at 22.5% regeneration.

This study demonstrates that adventitious shoot regeneration from callus induced at wound sites of shoot tip explants is the most efficient plant propagation method for R. laetum x aurigeranum. A system was developed for shoot regeneration from leaf explants of R. laetum x aurigeranum. Plant regeneration was also accomplished from leaf strips of commercial Rhododendron cultivars.

Micropropagation and *In Vitro* Plant Regeneration of Rhododendrons

by

Giovanni Iapichino

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed April 24, 1990

Commencement June 1991

APPROVED:

Professor of Horticulture in charge of major

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Date thesis is presented April 24, 1990

ACKNOWLEDGMENTS

I wish to express my sincere gratitude and appreciation to Dr. Tony H.H. Chen for his guidance, help and patience throughout the course of my graduate work.

I wish to thank my committee: Drs. Patrick Hayes, Leslie H. Fuchigami and Glenn Fisher for their assistance and advice. I particularly wish to thank Dr. Patrick Breen for encouraging me to come to Oregon State University for my graduate study.

I would also like to acknowledge the help and friendship of all my fellow graduate students.

Finally, a special thanks to Sharon Hope for taking the time to proofread this thesis.

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LIST OF ABBREVIATIONS: 1-H-Indole-3-acetic acid (IAA);
N-(3-methyl-2-Butenyl)-1H-purin-6 amine (2iP);
1-naphthaleneacetic acid (NAA);
1-H-indole-3-butyric acid (IBA);
2,4-dichlorophenoxyacetic acid (2,4-D).

MICROPROPAGATION AND *IN VITRO* PLANT REGENERATION OF RHODODENDRONS

CHAPTER 1

LITERATURE REVIEW

A. Introduction

Rhododendrons are normally propagated by means of seed, cuttings and grafting. Seeds often are used to propagate understocks and new hybrids. Many nurseries still propagate *Rhododendron* by stem cuttings, whereas grafting is primarily used for those species difficult to root. However, not all cultivars and species of *Rhododendron* can be easily rooted. Root regeneration from mature hardwood *Rhododendron* cuttings is often difficult (Kelly 1978). Young hardwood *Rhododendron* shoots are more easily rooted. Seasonal responsiveness may also have an important role in the rooting process. As in the case of deciduous *Rhododendron*, propagation can only be performed during a short period in the spring when there is a new flush of growing shoots (Fordham et al. 1985). Inducing new growth after rooting can represent a problem in some situations (Dirr and Heuser, 1987). Early studies by Anderson (1975)

demonstrated the feasibility of _in vitro_ techniques to propagate the cultivar _Rhododendron_ x "Rose Elf". Since then, the number of cultivars and species of _Rhododendron_ propagated by tissue culture is much larger and the number commercial companies involved in micropropagation of _Rhododendron_ has rapidly increased.

The advantages of micropropagation of _Rhododendron_ include:

a) A large number of plants can be produced in a small area, in a short time and all year round.
b) Eliminates the need for stock plant maintenance.
c) Increases the number of new selections and hybrids rapidly.
d) Improves propagation of species and cultivars considered difficult to propagate by conventional methods.
e) Produces and maintains pathogen free plant material.
f) Facilitates germplasm storage and exchange.

B. Propagation by _in vitro_ shoot axillary proliferation

There are five major factors which affect micropropagation of _Rhododendron_:

1. Type of explant material and technical problems associated with its surface sterilization.
2. Composition of the culture medium to establish and maintain axillary bud proliferation.

3. Types and concentration of growth regulators to induce rapid axillary bud growth.
4. Conditions for *in vitro* rooting and adaptation to soil environment.
5. Somaclonal variation.

1. Plant material

Culture establishment for most woody plants *in vitro* and particularly for *Rhododendron* is often considered to be more difficult than establishing cultures of herbaceous plants. Problems associated with proliferation in culture include 1) the relatively low response of woody plants to exogenous growth regulators, 2) the production of autotoxic exudates and 3) the failure of surface sterilization procedures to obtain aseptic cultures (Young et al., 1984).

In his early studies, Anderson obtained sterile cultures from *Rhododendron* seed. With improvement of the surface sterilization procedures, shoot tip explants were commonly employed. McCown and Lloyd (1983) used shoot tips two to four cm. long to establish *in vitro* culture of several genotypes of *Rhododendron*. Ettinger and Preece (1985) in *Rhododendron* x "P.J.M" hybrids observed that shoot tip explants 5 cm. in length were more successful than those 2 cm. in length. Moreover, the authors found it was easier to establish *in vitro* culture from shoot tips as opposed to single node explants.

Micropropagation of _Rhododendron_ requires efficient procedures to obtain sterile cultures. Once all leaves except those enclosing the apical meristem have been removed, shoot tips are normally surface sterilized by immersion for 20 minutes, with gentle agitation, in 20% clorox solution (1.05 % sodium hypochlorite) that contains few drops of Tween 20. The shoot tips are rinsed three times in sterile distilled water and placed in the shoot establishment medium (Kyte and Briggs, 1979).

Commercial micropropagation laboratories very often report that fungal and bacterial contamination is a serious problem with _Rhododendron_. The hairy and sticky surfaces of the explants are very difficult to disinfect. To overcome this problem, stock plants are frequently grown in a greenhouse; nevertheless, only 4-5% success is often achieved (Meyer, 1982). Anderson (1980) suggests that to resolve the difficulty experienced in decontaminating explants, material should be grown in media containing antibiotics and fungicides for a short period before it is transferred to new medium. A similar procedure has been proposed by Jones et al. (1977) to establish sterile apple cultures.

If a sterile culture is established, within 2 or 3 months axillary buds develop into new shoots. These shoots are subcultured into fresh medium supplemented with appropriate growth regulators to enhance shoot proliferation.

Subculturing is necessary every 4-8 weeks, depending on the species and cultivar to maintain culture uniformity and rapid growth.

2. Culture medium

Anderson (1975) was the first to describe a successful *in vitro* shoot multiplication protocol for *Rhododendron*. Anderson's success was achieved by revising the Murashige and Skoog (1962) inorganic formula (MS). Early failures in *Rhododendron* tissue culture were found to be associated with a MS medium toxic reaction to the explant at the explant-medium interface. Visual symptoms were stem browning, foliage chlorosis and eventual necrosis. These results were attributed to saline toxicity due to the presence of excess nitrogen and potassium, which are the major salt components of the MS medium. Anderson modified the MS formula by reducing KNO_3 from 1,900 to 480 mg/liter and NH_4NO_3 from 1,650 to 400 mg/liter (maintaining the same ratio of nitrate to ammonium nitrogen) and replacing KH_2PO_4 with NaH_2PO_4 (Anderson 1978 and Anderson 1984). Anderson also lowered the pH value of the medium from 5.7 to 4.5. The need for low salt medium has been tentatively related to the fact that rhododendrons are easily injured by salts in fertilization practice (Ma and Wang, 1978). Examples of other woody plants that have been successfully grown *in vitro* on lower salt concentrations than used by Murashige and Skoog are: almond (Tabachnik, 1977), conifers

(Cheng, 1978), apple (Wermer et al.1980), blueberry (Zimmerman, 1980), olive (Rugini et al.1981), aspen (Noh et al.1986).

Anderson's studies were confined to the propagation of a small number of related genotypes (Anderson, 1984). Further studies have demonstrated the suitability of the Anderson medium for shoot multiplication of many different Rhododendron species (Douglas, 1984; Ettinger and Preece, 1985; Fordham et al. 1982). However, in vitro propagation of rhododendrons has also been achieved in media other than Anderson's. For example, Preil and Engelhardt (1977) grew meristem tip explants of R. simsii successfully in MS medium at 1/10 strength with 2% sucrose. Both Rhododendron and Kalmia belong to the Ericaceae family. Another low salt medium, originally developed for Kalmia latifolia by Lloyd and McCown, (1980) and termed Woody Plant Medium (WPM), has proved to be an effective medium for the genus Rhododendron. Using WPM medium, McCown and Lloyd (1983) successfully propagated in vitro shoot tips of R. canadense, R. x Gibraltar, R. yedoense, R. schlippenbachii and R. mucronulatum. WPM medium has also been proved to be effective for micropropagation of R. calendulaceum and R. chapmanii (Blazich and Acedo, 1988; Blazich et al. 1986). Economou and Read (1984) have obtained optimum in vitro shoot proliferation in deciduous rhododendrons by further reduction of the KNO_3 originally included in Anderson

medium and by changing the ratio of $NH_4:NO_3$ from 1:2 to 1:1.

3. Plant growth regulators

Among the plant growth regulators, cytokinins have been found essential during the establishment stage of Rhododendron culture in order to induce and sustain axillary shoot growth. Cytokinins are also important in the proliferating stage to enhance multiple shoot formation (Anderson, 1984; McCown and Lloyd, 1982; Economou and Reed, 1984; Douglas, 1984; Ettinger and Preece, 1985). The beneficial effect of cytokinins with respect to inducing axillary bud growth has been related to its ability to lessen apical dominance imposed by auxin generated in actively growing meristematic tissues. Anderson (1984), after testing the response of four related Rhododendron cultivars to different concentrations of isopentenyladenine (2iP), concluded that the response is cultivar dependent. Different responses to 2iP were also found by Blazich and Acedo (1988) and by Blazich et al. (1986) respectively, in R. chapmanii and R. calendulaceum. These differences have been related to the extreme genetic diversity within the genus.

Using WPM medium McCown and Lloyd (1982) found 2iP was more effective than 6-benzylamino purine (BA) in stimulating axillary shoot proliferation in seven genotypes of Rhododendron. The absence of cytokinins in the media

resulted in rapid senescence of the explants. It was surprising to find that BA was detrimental in some genotypes. Similar results were reported by Dabin and Bouharments (1983) in R. sismii and by Anderson (1975) in his early studies on Rhododendron x "Rose Elf".

Economou and Read (1984) compared the effects of different concentrations of 2iP (5 to 20 mg/l) in stimulating axillary bud break in Minnesota deciduous rhododendrons. They found 2iP to be essential for in vitro shoot proliferation.

Fordham et al. (1982), using Anderson medium, compared the effects of different cytokinins on axillary and adventitious shoot proliferation in Exbury azaleas (Rhododendron spp.). The greatest number of shoots was obtained on Anderson medium containing zeatin or 2iP. BA caused stem and leaf necrosis. Similar negative effects caused by BA are reported by Norton and Norton (1984) and by Ettinger and Preece (1985) with Rhododendron x "PJM". However, different responses have been found in species of families other than Ericaceae, such as apple (Rosaceae) in which BA increases shoot proliferation (Lundergan and Janick, 1980). Norton suggested that Ericaceae probably have an enzyme to degrade BA by cleavage of the benzyl group from the N6 position, that results in the loss of cytokinin activity.

The role that auxin plays on in vitro axillary shoot proliferation of Rhododendron is not completely clear.

Anderson (1984) tested six different concentrations of IAA (0 to 8 mg/l) in combination with five different concentrations of 2iP (0 to 20 mg/l) on Rhododendron cultivars. Increasing IAA concentration was not effective in increasing the number of usable shoots per culture. McCown and Lloyd (1982) observed a negative effect of IAA in the proliferation medium due to callus induction and consequent formation of adventitious shoots. Similar negative effects of IAA associated with callus formation were observed by Preil and Engelhardt (1977) on different cultivars of R. sismii. In contrast, IAA at 1 mg/l in combination with 2iP at 10 mg/l was effective in enhancing shoot growth in Minnesota deciduous azaleas (Economou and Read, 1984). The relative effectiveness of other auxins in the propagation of Rhododendron is not known.

4. Rooting

Rooting of in vitro proliferated Rhododendron shoots can be accomplished by two procedures. 1) In vitro and 2) In vivo.

The in vitro procedure is very often accomplished by using growing conditions that differ from those of the multiplication stage. Anderson (1984) obtained 100% rooting in different cultivars of Rhododendron by using Anderson's shoot induction medium (Anderson, 1984) at 1/4 strength devoid of auxin. Douglas (1984) achieved 80 %

rooting *in vivo* using Anderson's medium without auxin. By adding indol butyric acid (IBA) to the medium he was able to increase the percentage of rooting up to 100%. The medium developed by Economou and Read (1984), MS medium at 1/4 strength, supplemented with 2 or 4 mg/l IAA or IBA proved to be effective for rooting proliferating shoots of *R. prinophyllum* (Dai et al.1987). IAA was also found to be effective in stimulating root proliferation in *R. sismii* (Preil and Engelhrdt, 1977).

Anderson in his early studies (Anderson, 1978) suggested elimination of the transition period in which the proliferating shoots are rooted *in vitro* before transfer to soil. His intention was to reduce tissue culture costs. In fact, rooting from shoots *in vitro* is the most labor intensive stage because it is the only step in which a large number of single shoots must be handled. In contrast, shoot clusters can be manipulated in the earlier stages of shoot proliferation. Moreover, a delay in growth has often been observed after the *in vitro* rooted plants are transferred to the soil. This has been related to root damage during manipulation aggravated by lack of induction of a good root hair system *in vitro* (Deberg and Maene, 1981). In contrast, direct rooting *in vivo* offers a saving in time and labor. Furthermore, roots initiated in the soil are immediately functional, more prolific and less subject to disease (Douglas, 1984). According to Anderson (1978), critical factors involved in the *in vivo* rooting

process are: 1) the shoot condition at the proliferating stage and 2) the porosity and relative humidity of the rooting environment. A soil mixture of peat and perlite (v:v = 1:1) and a relative humidity of 80% are suggested to assure high percentages of root induction (Anderson, 1978; McCown and Lloyd, 1982; Ettinger and Preece, 1985). Blazich (1986) recommends a soil mix of vermiculite and peat and a microcutting dimension >10 mm. to allow easy handling of _R. chapmanii_. The ease with which _in vivo_ _Rhododendron_ rooting takes place has been related to the tissues's reversion to juvenile traits since micropropagated plants have a morphological aspect similar to seedlings (Blazich et al., 1986).

5. Somaclonal variation

Clonal propagation by axillary shoot proliferation permits a high degree of genetic stability provided somaclonal variation does not occur. Albino/green chimeras have been detected by McCown and Lloyd (1982).

Ettinger and Preece (1985) have compared conventional propagated cuttings and micropropagated _Rhododendron_ x "PJM" plants. They observed that whereas the first type had more lignified stems and vertical habit, the second type assumed a horizontal growth habit. Furthermore the latter showed a certain level of basal branching which occurred after the plants assumed a horizontal growth.

Basal branching has been also observed by McCown and Lloyd (1982) in Rhododendron and has been related to a carry over effect from growth regulators supplied to the cultures in the previous stages. The base of the stem may be weakened with some Rhododendron cultivars propagated by tissue culture. This phenomenon may lead to loss of commercial value such as reported by Sudkamp and Lohr (1989). A constricted region at the base of the stem has been identified, however, the cause of this malformation is unclear.

C. Propagation by adventitious shoot proliferation

Adventitious shoots are those shoots induced to form on tissues which normally do not produce these organs. They can be produced directly on the explant or on a callus derived from the primary explant. The production of adventitious buds represents one technique for large scale clonal propagation, provided that a high frequency of somaclonal variation does not occur. Adventitious buds have been observed by McCown and Lloyd (1982) on shoot tip cultures of different Rhododendron genotypes originally yielding axillary shoots. Shoot tip explants were placed on WPM medium supplied with 2iP or BA. Adventitious shoots were identified by their later emergence into the shoot mass and by their small size. Adventitious shoots were also reported by these authors on callus developed at the

basal end of the shoot tip. Adventitious shoot proliferation is reported by Fordham et al. (1982) on the abaxial and adaxial sides of leaves from Exbury azalea shoot tip explants grown on Anderson's medium supplemented with 2iP or zeatin. In contrast, leaves cultured separately from the stem showed necrosis and only a limited number were able to produce adventitious buds. Imel and Preece (1988) succeeded in inducing adventitious shoot formation at the petiole end of leaves exposed to Anderson's medium supplied with 2iP or thidiazuron in combination with IBA. They found a pretreatment of the leaves on the same medium supplemented with 50 uM of 2iP and 10 uM of IBA to be critical for leaf survival and for further regeneration after explant transfer to the experimental medium. Thidiazuron was found more effective than 2iP in inducing adventitious shoot formation.

As mentioned above, one of the most important problems encountered in Rhododendron micropropagation is the difficulty in establishing an aseptic culture through shoot tip explant surface sterilization. This problem has caused some investigators to search for alternative explant sources for successful micropropagation of Rhododendron. Flower buds in Rhododendron are covered by scales and by an additional papery white covering structure which protects the cluster of florets that will form the inflorescence. Thus, they are a good source of explants because of their lower level of contamination. Other advantages of using

flower buds for tissue culture include a longer season for collecting explants. In Rhododendron, flower buds are formed in late fall and remain unopened until spring time. Thus, flower buds are available for 5-6 months, whereas shoot tip explants are only available for 1-2 months in the spring. Furthermore, using flower bud explants for tissue culture results in less damage to the stock plants since the vegetative shoots are not removed. Meyer (1982) has successfully used flower buds for the micropropagation of R. catawabiense. After the normal procedure of sterilization he excised single florets trying to maintain as much pedicel as possible, because callus would originate from it and from the ovary base. By adding IAA at 1 or 4 mg/l and 2iP at 5 or 15 mg/l to Anderson's medium, Meyer was able to produce callus after 6-8 weeks. A period of two weeks in the dark before transferring the callus to the light in Anderson's medium supplemented with lower levels of IAA and 2iP seemed to enhance proliferation of adventitious shoots. Rooting was then accomplished by exposing the shoots to a fresh medium supplied with 4 mg/l of IAA or by transferring them to a sterile mixture of sand, perlite and peat.

Day et al. (1987) have investigated the use of ovary cultures as a source of explants in the micropropagation of R. prinophyllum which is difficult to propagate by conventional methods and by in vitro shoot tip proliferation. They used the same techniques proposed by

proliferation. They used the same techniques proposed by Meyer, except that the intact florets or excised ovaries were placed horizontally on Anderson's shoot induction medium (Anderson, 1984) supplemented with 1 or 4 mg/l of IAA and 4 or 15 mg/l of 2iP. After 2 weeks of culture the petals of the former explant separated naturally and were excised. Many white protrusions arose from the ovary walls, from the ovary wall calli and from the base of the pedicels. A period of darkness (one month) accelerated the regenerative process. Leaving the pedicels increased ovary survival by decreasing the risk of injury and desiccation. The protrusions became green and bud primordia differentiated from them when the cultures were exposed to light. The shoots were then transferred into Economou shoot propagation medium (Economou and Read, 1984) supplemented with 1 mg/l of IAA and 5 mg/l of 2iP. Thereafter, new shoots proliferated and shoot clusters were transferred to fresh medium. By this technique hundreds of shoots were produced from one ovary and later successfully rooted on a rooting medium. The use of flower buds as a source of adventitious bud proliferation permits the researcher to obtain hundreds of new plantlets from a single explant. However, since shoots are produced from ovary and callus cells, some possibility of somaclonal variation exists. This aspect should be carefully evaluated before using the technique for mass propagation. Aberrant plant types were detected by McCown and Lloyd (1982) among

Rhododendron plants obtained from the callus at the basal end of primary shoot tip explants. In regard to this last aspect, Harbage et al. (1987) pointed out the necessity to verify the stability of *Rhododendron* derived from callus. They first investigated the initiation, maintenance and subsequent organ regeneration potential of *Rhododendron* callus in two Exbury Hybrids. For the induction and maintenance of callus, shoot tips were excised from an *in vitro* proliferating shoot tip culture. Shoot sections, after tip and leaf removal, were placed horizontally in Anderson's medium supplied with one concentration of zeatin in combination with three different levels of 2,4-D for two 3 week culture cycles. Within 2 weeks all cultures produced callus. However, the lowest and intermediate concentration of 2,4-D produced both callus and adventitious buds whereas the cultures supplied with the highest level of 2,4-D produced only callus. Regeneration was accomplished by transferring the cultures into fresh medium supplied with IAA and zeatin. Zeatin was found to be essential for adventitious bud regeneration. A negative correlation was found between the number of subcultures to which the callus had been subjected and shoot regeneration. In fact, after 12 subculturing cycles the number of shoots produced from callus declined from 70 to 0 in one cultivar and from 30 to less than 10 in the other. Decline in morphogenetic potential from callus by repeated

subculturing has been thought to be associated with chromosome aberration aggravated by 2,4-D (Yeoman and Forche, 1980). This suggests that plant material obtained from adventitious shoots regenerated from callus should be carefully screened for stability.

D. Conclusions

In vitro propagation of *Rhododendron* has become a successful alternative to conventional methods of vegetative propagation. Anderson's and WPM medium have been satisfactorily used to micropropagate several species of *Rhododendron*. However, due to the different requirements in medium composition and growth regulator among species and/or cultivars, a particular protocol has to be developed to fit the plant material of interest. For example, the exogenous cytokinins required to induce axillary shoot growth vary both from species to species and from cultivar to cultivar. Modifications to the MS medium reported by Anderson (1984) as well as by Lloyd and McCown (1983) were related only to macronutrient concentrations. Macronutrients are mainly involved with nutrition and growth of the explant in culture. Micronutrients influence both growth and development of plant *in vitro*. Therefore, further studies on micronutrient composition and concentration of the medium might improve the *in vitro* response of *Rhododendron*.

Most reports of tissue culture with Rhododendron are limited to micropropagation by axillary shoot proliferation. However, a much greater number of plants might be produced by induction of adventitious shoots from callus or somatic tissues, provided that somaclonal variation does not occur. This method would be a reliable alternative in those species where axillary shoot proliferation is limited. Furthermore the ability to regenerate whole plants from somatic tissues such as leaf sections is a prerequisite for Agrobacterium mediated transformation of higher plants. Therefore, the development of methods for plant regeneration from somatic tissues of Rhododendron would facilitate the feasibility of transformation in this species.

CHAPTER 2

MICROPROPAGATION OF A VIREYA HYBRID OF RHODODENDRON

A. Introduction

The genus Rhododendron, section Vireya comprises about 300 species endemic to south-east Asia (Sleumer, 1966). Almost half of the 300 Vireya species, including the species R. laetum x aurigeranum, are native to New Guinea which lies just south of the Equator between 2° and 12° latitude south. According to the Royal Horticultural Society, Vireya rhododendrons are classified as "normally requiring greenhouse protection during the winter months" (Royal handbook, 1980). Those species native to low altitudes in New Guinea are irreparably damaged if exposed, even for a short time, to temperatures below 0° C (Withers, 1984). Since Vireya rhododendrons do not require a resting period, they may flower at any time of the year. Therefore, they may have great potential as greenhouse ornamental plants. Moreover, they represent a source of breeding material for the improvement of hardy rhododendron flower quality. Vireya flowers range in size from less than 1 cm. to 10 cm. in length and may be found in a variety of colors from white to yellow, from orange to deep red; the flower has diverse shape from tubular form to semi-open bells

(Williams and Rouse 1983). Within the section Vireya the hybrid R. laetum x aurigeranum deserves particular attention because of its long lasting "golden yellow" flowers which are not found in most common cultivated hardy rhododendrons.

Many species of Rhododendron are currently propagated by tissue culture (Chee, 1985). However, there is no report in the literature concerning the micropropagation of Vireya species. The development of an efficient micropropagation system would facilitate mass propagation of these species. The objective of this research was, therefore, to develop a protocol for the propagation of the Vireya hybrid laetum x aurigeranum.

B. Materials and Methods

Experiment I. Establishment of shoot tip culture.

Explants for the establishment of aseptic culture were collected from stock plants of the Vireya hybrid laetum x aurigeranum maintained under controlled greenhouse conditions. Stock plants were grown in 22 cm. plastic pots containing a mixture of perlite, peat and soil (v/v/v=1:1:1). A controlled release fertilizer (Osmocote 14-14-14) was incorporated into the soil mixture at the time of planting. Greenhouse temperatures ranged from 26 to 32 ^{o} C. Plants were watered as needed to keep the

medium moist.

Actively growing apical shoot tips, 7-8 cm. in length, were collected from the stock plants. All leaves except those immature leaves enclosing the apical meristem and the leaf primordia were removed. Shoot tips were rinsed under running tap water for 20 minutes. The explants were submersed for 2 minutes in 70 % ethanol solution followed by soaking for 40 minutes with gentle agitation in 20% Clorox solution containing 20 drops/liter of Tween 20 (final solution containing 1.05 % sodium hypochlorite). After rinsing 3 times in sterile distilled water the basal end was removed and shoot tips (ca.5 cm. long) were placed horizontally on the surface of media.

Explants were cultured on revised Anderson's medium (Anderson, 1984) containing 30 g/l sucrose, 100 mg/l inositol, 8 g/l Difco Bacto agar and 80 mg/l adenine sulphate. A factorial experiment with various combinations of IAA (0 to 8 mg./l) and 2iP (0 to 25 mg/l) concentrations was used to determine the optimal growth regulator combination for the production of axillary shoots.

Growth regulators were added to the media prior to autoclaving. After adjusting the pH to 4.5 and adding agar, media were autoclaved at 121 C for 20 minutes. Fifty ml of medium were dispensed into each sterilized Magenta GA7 vessel. Explants, one per vessel, were transferred into each of the various media tested. Vessels were sealed

with Parafilm and incubated at 25 $\pm$1 C with a 16 hour photoperiod and 50 uE $m^{-2}S^{-2}$ light intensity. A randomized block design was applied. Five vessels were used for each treatment combination. Treatments were replicated three times over time. Cultures were evaluated for the number of usable axillary shoots (> 2 mm.) after three months of culture.

Experiment II. Shoot proliferation from in vitro multiplied shoots.

The first part of this experiment was performed to identify any possible carry over effect of the growth regulators used in the establishment medium on the subsequent stage of shoot proliferation. Main and axillary shoots that sprouted from the primary shoot tip explants were removed, counted and marked separately according to the establishment media to which the original shoot tip explants had been exposed in the previous experiment. Individual shoots were subcultured onto fresh Anderson's medium, containing IAA, 4.0 mg/l, and 2iP, 15 mg/l. Shoots were placed into each of sterilized Magenta GA 7 vessels containing fifty ml of the medium. Preparation of the medium and growth conditions were identical to those used in the shoot tip culture establishment experiment, except that only one growth regulator combination was used. According to the experimental design applied in

experiment I, a randomized block design with five vessels for each type of shoots was used. Treatments were replicated three times over time. Cultures were evaluated for the number of usable shoots (>2 mm.) after three months of culture.

We observed in the previous experiments that callus tissue developed at the basal end of the primary shoot tip explants and/or at the cut surface where leaves and axillary shoots were removed (Fig.2.1B and C). Those calli were brownish in color. After about 6 months in culture adventitious shoots proliferated from these calli.

The calli with multiple shoots (clumps) were divided into small pieces (0.5 cm. in diameter) and subcultured monthly into Anderson's medium supplemented with 4 mg/l of IAA and 15 mg/l of 2iP. Adventitious shoots proliferated rapidly and provided material for the second part of experiment II.

Individual adventitious shoots, ranged from 2-10 mm., were excised from callus clumps and placed into 10 x 60 mm. petri dishes containing 10 ml of Anderson's medium supplemented with various combination of IAA and and 2iP concentrations. A completely randomized design was used, with 5 replicates per treatment. A replication was defined as a petri dish containing 4 single shoots. The number of newly formed shoots was recorded after 6 months of culture.

Experiment III. Rooting and establishment in the soil.

To determine the optimal conditions for *in vitro* rooting of *in vitro* generated shoots, a complete combination of four concentrations of Anderson's medium (1, 1/2, 1/4 and 1/8 strength) was used in a factorial experiment with five IAA concentrations (0, 0.1, 1.0, 5.0 and 10 mg/l). Using the optimal ionic strength, the rooting ability of shoots cultured on media containing the optimal IAA concentration was compared against equal molar concentrations of different auxins (2,4-D, IBA and NAA).

In both experiments a completely randomized design was used with 5 replicates. A replication consisted of a petri dish containing 9 adventitious shoots. Data were recorded as percent of shoots rooted after 4 weeks, number of roots per shoot and root length. Percentage data were subjected to arcsin transformation prior to statistic analysis.

In order to verify possible effects of the rooting media by means of plantlet adaptation to the soil conditions, rooted plantlets were separated according to the rooting treatments. Plantlets were transplanted into compost (Jiffy mix) in seed trays and covered with clear plastic bags to maintain a high relative humidity. The compost was kept moist by frequent watering and seed trays were kept in a greenhouse under the same growth conditions as the stock plants. Ventilation of the plantlets was increased with

time by increasing the size of holes made on the bags and after one week the plastic bags removed. Three weeks after transplanting the percentage of survival was evaluated.

C. Results

Establishment of shoot tip culture.

Although the greatest number of axillary shoots was induced on medium supplemented with 4 mg/l IAA and 15 mg/l 2iP (5.3 shoots per shoot tip explant) we did not find a significant interaction between IAA and 2iP concentrations. Furthermore we found no need for IAA in the medium to induce axillary bud break. Axillary buds started to grow after 1 month of culture and completely developed after three months of culture (Fig.2.1 A). The effect of different concentrations of IAA and 2iP on axillary shoot proliferation are shown in Table 1. Regardless of IAA concentration, 1.0, 1.2, 1.4 and 3.8 shoots per shoot tip explant respectively developed on Anderson's medium supplied with 0, 5, 10 and 15 mg/l 2iP. 2iP concentrations of 15 mg/l significantly induced axillary bud proliferation whereas, levels of 2iP lower than 15 mg/l only stimulated main shoot growth. In another test, data not shown, higher concentrations of the same growth regulators (IAA up to 8 mg/l and 2iP up to 25 mg/l) did not show significant differences in the number of axillary shoots developed nor in the time required to establish the cultures.

Shoot proliferation from in vitro multiplied shoots.

To determine the possible carry-over effect of the growth regulators used to establish the shoot tip cultures, main and axillary shoots from a previous experiment were subcultured onto Anderson's medium, supplemented with 4 mg/l IAA and 15 mg/l 2iP. After three months of culture the majority of axillary buds present at the leaf axis of these shoots remained quiescent (Table 2.2.; Fig.2.2A.). When comparing the data presented in Table 2.1. and Table 2.2., it was found that: 1) regardless of the growth regulator combination used for establishing shoot cultures, subculturing increased the number of shoots per shoot explant ; and 2) media supplemented with 4 mg/l IAA and 15 mg/l 2iP gave the highest proliferation rate (11.7 shoots per axillary shoot).

It was observed that a callus-like structure developed at the base of the shoots, followed by the differentiation of shoot primordia (Fig.2.2A.). These shoot primordia developed and consisted of a combination of leaflets and adventitious shoots. Shoots were difficult to count and to separate. Although it was difficult to quantify, generally shoots which had been obtained from primary shoot tips exposed to media, supplied with 15 mg/l of 2iP, produced a significantly larger number of new shoots than those which had been obtained from explants established on media

had been obtained from explants established on media supplemented with either 0, 5, or 10 mg/l of 2iP respectively.

Shoots developed from callus proliferated from the primary explants.

Primary explants originally placed on medium supplied with 4 mg/l of IAA and either 10 or 15 mg/l of 2iP, after axillary shoot removal, were left for several months on the exhausted medium. Callus formation occurred at the basal end of the original shoot tip and from the stub of the excised leaves and axillary shoots (Fig.2.1B). The calli were friable and brown in color. After about 6-7 months of culture, a large number of adventitious shoots was produced from the calli clumps (up to 70 shoots per explant. Fig. 2.1C.). Clumps of adventitious shoots with small amounts of callus (Fig.2.2B.) were divided and subcultured onto fresh medium supplied with 4 mg/l IAA and 15 mg/l 2iP. This resulted in greater shoot proliferation (about 25 shoots per clump. Fig.2.2C.).

Shoot proliferation from adventitious shoots.

Individual shoots were excised from clumps of adventitious shoots and transferred into Anderson's medium supplemented with IAA (2 to 6 mg/l) and 2iP (10 to 30 mg/l) in a factorial combination (Table 2.3.). After two months of culturing the base of the shoots developed a small amount

of callus first and then green primordia appeared. The primordia differentiated into small leaves. Only after 6 months of culture could new shoots could be distinguished as they emerged from the mass of leaves (Fig.2.3.). There was no significant difference between 2iP treatments nor was there any interaction between the growth regulators. However, IAA at 6 mg/l was found to be effective in inducing new shoot formation from the basal end of single shoots (7.4 shoots per single shoot) compared to lower levels of IAA .

Rooting and establishment in the soil

In vitro rooting of excised shoots was accomplished in about 1 month. Different ionic concentrations of Anderson's medium in combination with different concentrations of IAA influenced the rooting of shoots (Table 2.4.). Absence or low levels of IAA (< 5 mg/l) in combination with full strength Anderson's medium resulted in no rooting. By increasing IAA concentrations and lowering the ionic concentration the percentage of rooted plants (Fig.2.2D and E.) was significantly increased. The highest value of *in vitro* rooted shoots was obtained on medium with 1/4 strength supplied with 5 mg/l of IAA (86.7%). We also observed that ionic concentrations lower than 1/4 strength of Anderson's medium in combination with IAA concentration > than 5 mg/l did not show significant differences in the percent of rooting.

Significant differences were found with respect to the number of roots per shoot among treatments. The number of roots induced from shoots exposed to Anderson's medium at 1/4 strength supplied with IAA at 5 mg/l (6.2) differed significantly from that obtained in all the other treatments (Table 2.5.). Root length was less influenced by the treatments tested (Table 2.6.).

Testing equal molar concentrations of different auxins (equivalent to 5 mg/l IAA), added to Anderson's medium at 1/4 ionic strength, provided further indications to optimize *in vitro* rooting of adventitious shoots (Table 2.7.). IAA was significantly more effective than IBA and NAA in inducing rooting. Only callus formation was induced in media containing 2,4-D. Root length was similar for the IAA, NAA and IBA treatments.

Observation of the plantlets three weeks after transplanting into the soil showed 100 % survival regardless of the *in vitro* rooting media (Fig.2.2F.).

New roots generated first from the root system already developed *in vitro* and later from the base of the plantlets.

D. Discussion

We observed three types of shoot multiplication:
1) Axillary shoot induction (Fig.2.1A.). 2) Organogenic callus was induced at the cut surfaces of the primary shoot

tip explants (Fig.2.1C.). Subsequent subculturing of clumps of shoots with callus induced massive production of adventitious shoots (Fig. 2.2C.). The efficiency of this method is much higher than the previous type. 3) When single excised adventitious shoots were cultured into fresh medium, a low rate of multiplication was achieved (Fig.2.3.). The limit of this method is that a longer time is needed to obtain multiple shoot formation.

The common method of micropropagation of Rhododendron relies on the proliferation of axillary shoots when they are subcultured into fresh medium (Kyte and Briggs, 1979). This cycle can be repeated indefinitely and allows the production of true type and uniform plant material, since production of adventitious shoots is avoided. In this study, however, majority of the axillary shoots were not able to proliferate after subculturing into fresh medium containing 4 mg/l IAA and 15 mg/l 2iP. In contrast, new shoots primordia differentiated at the base of the explants. After three months of culture these primordia developed into a combination of leaflets, a few basal axillary shoots and adventitious shoots and had a moss-like appearance. These shoots were difficult to handle, and showed a slow rate of growth. We estimated a 137 fold increase in shoot number in six months of culture. These observations suggest that the common protocol used to micropropagate the majority of Rhododendron species can not

directly be applied to the Vireya rhododendrons.

The terminal portion of the explant from the original shoot tip is often removed to enhance axillary shoot proliferation with in vitro culture of rhododendrons (Blazich et al.1986; Blazich and Acedo, 1988; Anderson, 1984). This procedure could not be applied to Vireya rhododendrons for two reasons. 1) The majority of the axillary shoots (4-5 per shoot) in mature plants of Vireya rhododendrons are generally clustered around the shoot tip apex. In preliminary experiments we observed that it is very difficult to remove the main shoot without damaging the axillary buds. 2) Our data showed that by manipulating the concentration of 2iP multiple shoot formation occurred satisfactorily without removal of the main shoot.

The presence of 2iP was essential to induce axillary shoot growth in R. laetum x aurigeranum, and increasing 2iP concentrations up to 15 mg/l enhanced the number of axillary shoots per explant at all IAA concentrations. Similar results were observed by Anderson (1984) in the cultivar "Scintillation", Fordham et al. (1984) in Exbury azaleas (Rhododendron spp.) and Douglas (1984) in several cultivars of Rhododendron widely grown in Europe. Conversely, lower levels of 2iP are reported to be effective for some rhododendrons by McCown and Lloyd (1982) and by Blazich et al. (1986). The function of 2iP and other cytokinins is thought to reduce apical shoot dominance and consequently induce axillary shoot

proliferation (Economou and Read, 1984). The different requirements among different species and cultivars of Rhododendron may be explained on the basis of variations in endogenous levels of cytokinins or the difference in metabolism of the cytokinins within the tissue explant.

Anderson (1984) and McCown and Lloyd (1982) reported that IAA and 2iP concentrations above certain limits are not effective and/or can be detrimental in inducing Rhododendron multiple shoot formation. This is consistent with our results using R. laetum x aurigeranum. In fact, IAA and 2iP concentrations higher than 4 and 15 mg/l, respectively, did not increase the number of axillary shoots.

As mentioned before, axillary buds of R. laetum and aurigeranum are at the apex of the shoot tip explant. These buds remain correlatively inhibited unless an exogenous stimulus is provided (as for example by culturing them in vitro in the presence of cytokinins). The growth habit of these axillary shoots developed in culture, appears to be different from that which occurs in the mature plants. Internodes are short and consequently leaf arrangement along the stem of these shoots is compact. The second type of shoot multiplication was obtained from adventitious shoot formation from callus. Adventitious shoot formation appeared from the clumps of callus at the cut surfaces of the primary shoot tip explants (Fig.2.1B

and C.) cultured on media containing IAA 4 mg/l and 2iP 10 or 15 mg/l. Similar observations were made by McCown and Lloyd (1983) on R. Canadense, R.x "Boule de Neige" and R. x "PJM". Once adventitious shoot formation was induced from this callus, clumps of 4-5 shoots with small amount of callus (5 mm. diameter) were subcultured into fresh medium. After three months these clumps increased in size with an average of 40 shoots per clump (Fig.2.2B.), by dividing each clump and subsequently transferring them to fresh medium (Fig.2.2C.). We estimated that once organogenic callus is induced, a 1600 fold increase in shoot number in six months can be obtained. This protocol represents the most efficient method of micropropagating the hybrid R. laetum x aurigeranum. However uniformity and genetic stability of the plant material must be carefully evaluated before such a propagation method can be adopted.

The third type of shoot regeneration tested involved single adventitious shoots, devoid of callus, that were excised and cultured on medium containing IAA (6 mg/l) and 2iP (30 mg/l). A small amount of callus developed at the base of these shoots and later new shoots proliferated. We observed that the average number of shoots per single shoot obtained in this experiment (about 6 shoots per cultured shoot) was considerably smaller than that obtained when clumps of shoots with small amounts of callus were routinely subcultured (about 40 shoots per cultured shoot) to increase the plant material. We estimated that a 7.8

fold increase in shoot number occurred by means of this method.

The low salt medium developed by Anderson has been recognized as a suitable medium for several Rhododendron spp., as well as for other Ericaceous species (Fordham et al. 1982; Anderson, 1984; Ettinger and Preece, 1985; Harbage and Stimart 1987; Blazich et al. 1986; Blazich and Acedo 1988). We found that Anderson's medium was also good for establishing and maintaining an in vitro culture of the Vireya hybrid R. laetum x aurigeranum.

Root initiation is generally promoted by high auxin levels. Anderson (1984) observed that lowering the ionic strength of modified Anderson's medium to 1/4 strength resulted in increasing the percentage of in vitro rooting with several cultivars of Rhododendron. We found that the low ionic salt concentration of the medium and IAA concentrations interact significantly to induce in vitro root formation in R. laetum x aurigeranum. It is also evident that a critical level of IAA must be reached before root induction can occur. However, above this level IAA appeared to be detrimental since the rooting percentage and the number of roots per shoot decreased.

The other auxins tested were less effective than IAA in promoting root formation. 2,4-D is known to suppress organ differentiation (Gresshof, 1984) and in our study only induced callus. NAA is considered a more potent auxin

(Murashige and Skoog 1962) than IAA and its negative effect observed herein could be related to this property. IBA induced 50 % rooting, however, we noticed a tendency for an increased number of roots per shoot which could enhance the ability of the plantlets to adapt to the soil conditions.

By maintaining a high level of humidity in the environment *in vitro* rooted plantlets easily adapted to soil conditions. We obtained 100 % survival regardless of ionic strength and IAA concentration of the *in vitro* rooting medium.

A successful *in vitro* multiplication protocol has been developed for *Rhododendron laetum* x *aurigeranum*. Among the three types of shoot multiplication, adventitious shoot multiplication from organogenic callus, induced at the cut surfaces of the primary shoot tip explants, was the most efficient. The *in vitro* propagated plants are under evaluation for their genetic uniformity and clonal fidelity.

Fig. 2.1. A - Axillary shoot development in a shoot tip taken from greenhouse-grown plants of R. laetum x aurigeranum. The shoot was cultured on Anderson's medium with 4 mg/l IAA and 15 mg/l 2iP (establishment medium) for three months. B - Callus induction at the cut surfaces of a shoot tip explant after four months of culture on the establishment medium. C - Adventitious shoot proliferation from callus induced at the cut surfaces of a primary shoot tip after 6 months culture on the establishment medium.

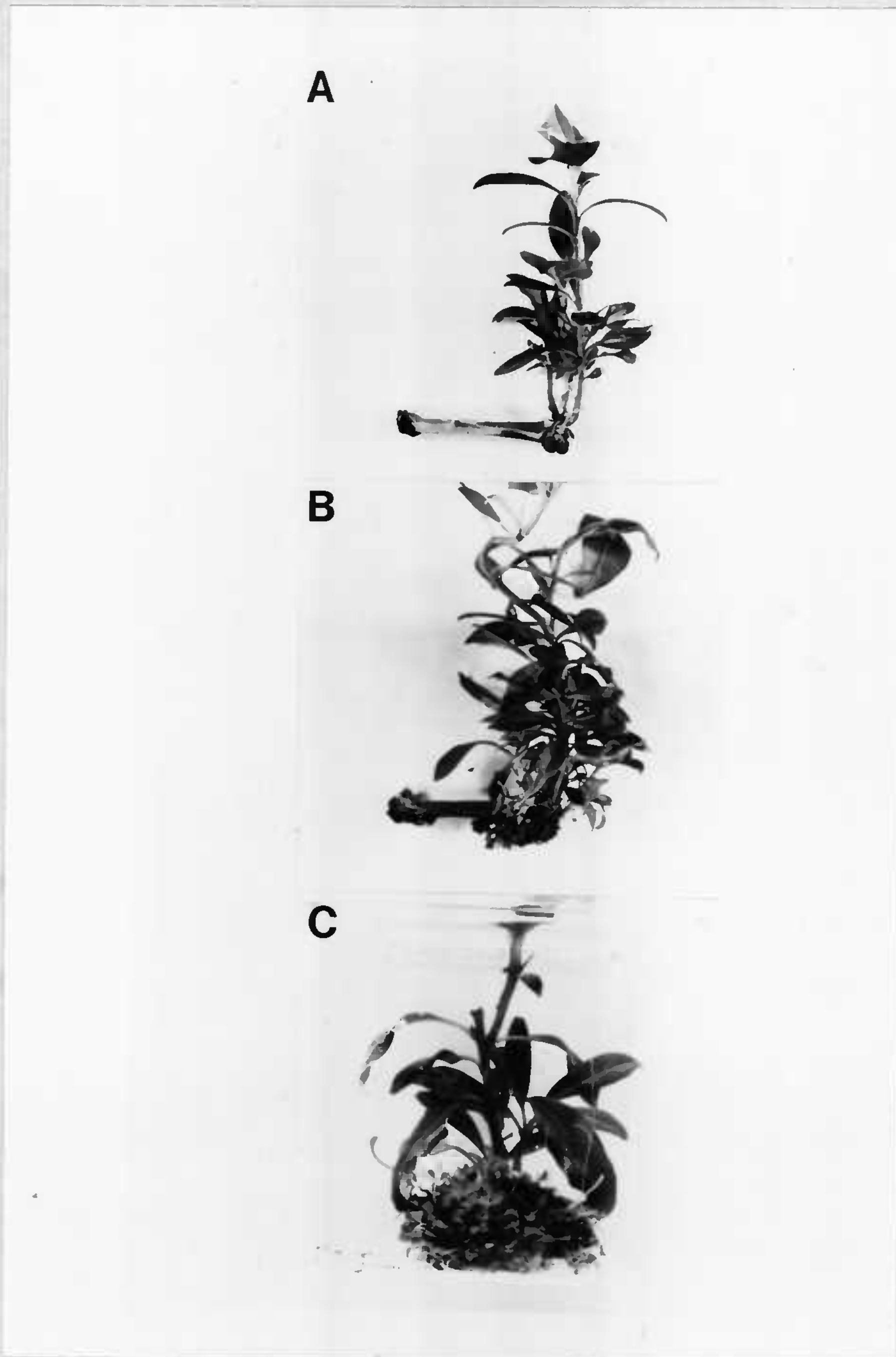

Figure 2.1.

Fig. 2.2. A - Organogenic callus induced at the base of axillary shoot cultured on Anderson's medium supplemented with IAA and 2iP. B - Cluster of shoots with small amount of callus proliferated in fresh Anderson's medium with 4 mg/l IAA and 15 mg/l 2iP. C - Proliferation of organogenic callus clumps consisted of adventitious shoots and callus tissues. D - *In vitro* rooting of adventitious shoots on 1/4 strength Anderson's medium supplemented with 5 mg/l IAA, after 1 month in culture. E - Plantlets with regenerated root systems before transfer to the soil. F - Micropropagated plants 2 months after transplanting in the soil.

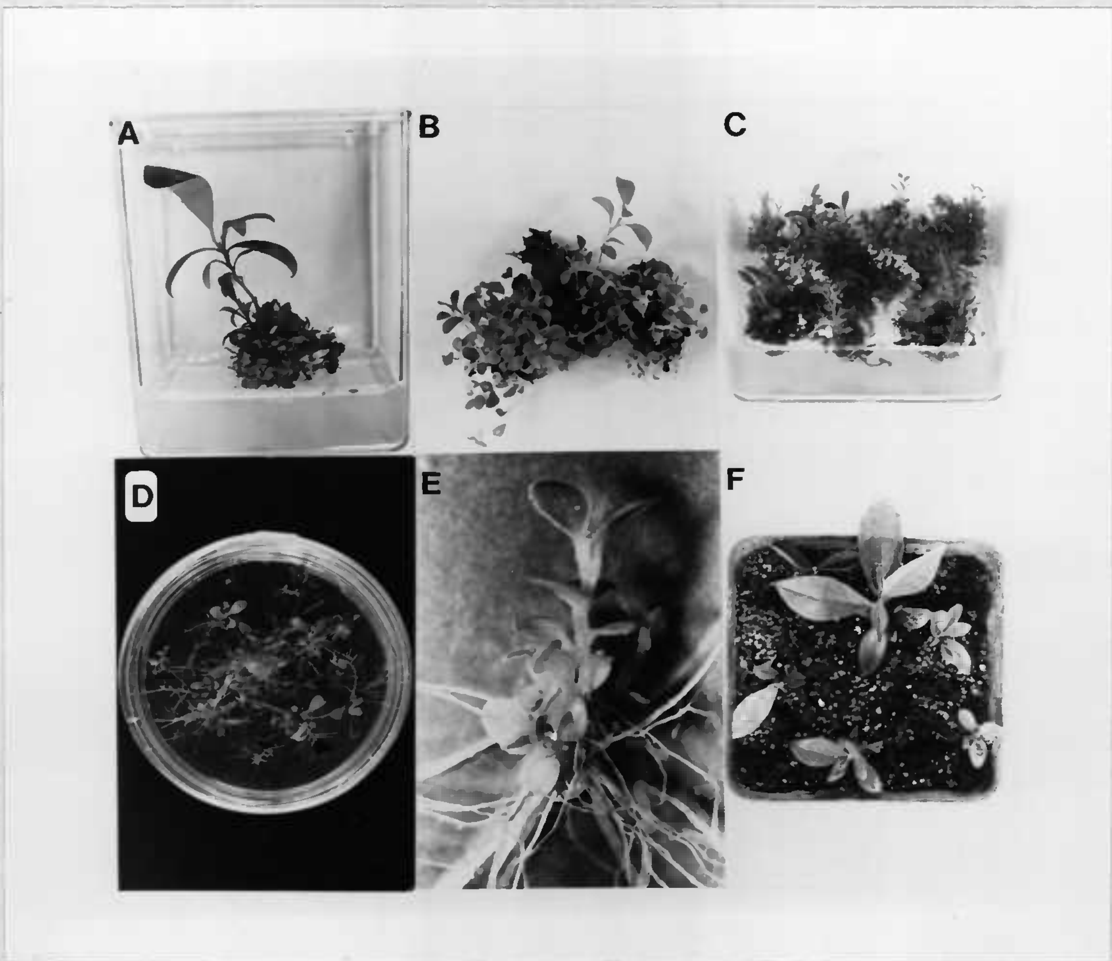

Figure 2.2.

Fig. 2.3. Adventitious shoot formation at the base of individual adventitious shoot (arrow) after 6 months of culture on Anderson's medium supplemented with IAA and 2iP.

Figure 2.3.

Table 2.1. Effect of different combinations of IAA and 2iP concentrations on *in vitro* axillary shoot proliferation in *R. laetum* x *aurigeranum* shoots taken directly from greenhouse-grown plants.

2iP (mg/l)	IAA (mg/l) 0	2.0	4.0	Mean[z]
		number of shoots per explant[y]		
0	1.0	1.1	1.0	1.0 b
5	1.2	1.1	1.3	1.2 b
10	1.4	1.7	1.1	1.4 b
15	2.5	3.7	5.3	3.8 a
Mean	1.5 a	1.9 a	2.2 a	

[z]Mean separation within columns and rows by Fisher's protected LSD (P=0.01).

[y]The number of shoot per explant after 3 months in culture.

Table 2.2. Effect of the IAA and 2iP combinations on shoot proliferation from shoots which had been established in culture. The axillary shoots, generated from shoots taken from greenhouse grown plants, were subcultured on Anderson's medium supplemented with 4 mg/l IAA and 15 mg/l 2iP. The number of newly proliferated shoots was recorded 3 months after subculturing.

	IAA (mg/l)			
2iP (mg/l)	0	2.0	4.0	Mean
	Number of shoots per explant[z]			
0	3.4	4.7	3.9	4.0 b
5	6.6	4.9	4.5	5.3 b
10	4.0	8.0	4.4	5.5 b
15	7.7	8.0	11.7	9.1 a
Mean	5.4 a	6.4 a	6.1 a	

[z]Mean separation within columns and rows by Fisher's protected LSD (P=0.01).

Table 2.3. Effect of different IAA and 2iP concentrations on shoot proliferation at the basal end of shoots after 6 months in culture.

2iP (mg/l)	IAA (mg/l)			Mean
	2.0	4.0	6.0	
	number of shoots/shoot explant[z]			
10	4.9	4.0	7.9	5.6 a
15	5.2	6.4	7.0	6.2 a
20	5.3	6.2	7.0	6.2 a
30	4.2	7.0	7.8	6.3 a
Mean	4.9 b	5.9 b	7.4 a	

[z]Mean separation within columns and rows by Fisher's protected LSD (P=0.01).

Table 2.4. Effect of ionic strength and IAA concentration on root regeneration after one month of culture[z].

Ionic strength	IAA (mg/l)				
	0	0.1	1.0	5.0	10
			Rooting %[y]		
1	0 g	0 g	0 g	20.4 de	8.9 efg
1/2	4.0 g	7.7 efg	30.8 d	34.0 cd	64.4 ab
1/4	8.0 g	23.5 def	60.7 bc	86.7 a	75.1 ab
1/8	42.2 cd	71.1 ab	33.3 d	73.3 ab	75.5 ab

[z]Mean separation by Fisher's protected LSD (P=0.05).

[y]Percentage data were arcsin-transformed before analysis. Untransformed means are presented.

Table 2.5. Effect of ionic strength and IAA concentration on the number of roots per shoot after 1 month of culture[z].

Ionic strength	IAA (mg/l)				
	0	0.1	1.0	5.0	10
	number roots per shoot				
1	0 fg	0 fg	0 fg	1.3 def	0.1 fg
1/2	0.4 fg	0.9 efg	1.7 cde	3.2 b	2.3 bcd
1/4	0.3 fg	2.1 bcd	2.6 bc	6.2 a	3.5 b
1/8	1.6 cde	2.9 b	1.3 def	2.4 bc	2.0 bcd

[z]Mean separation by Fisher's protected LSD (P=0.01).

Table 2.6. Effect of ionic strength and IAA concentration on root length (cm) after 1 month of culture.[z]

Ionic strength	IAA (mg/l)				
	0	0.1	1.0	5.0	10
	Root length (cm.)				
1	0 d	0 d	0 d	1.2 a	0.2 cd
1/2	0.7 abcd	0.3 bcd	0.6 abcd	0.7 abcd	1.0 ab
1/4	0.1 d	0.7 abcd	0.8 abc	1.0 ab	0.8 abcd
1/8	0.7 abcd	0.9 abc	0.7 abcd	0.8 abcd	0.7 abcd

[z]Mean separation by Fisher's protected LSD (P=0.01).

Table 2.7. Effect of different auxins on root regeneration after 4 weeks of culture. (Medium: Anderson at 1/4 strength)[z]

Auxin	Rooting[y] %	No.roots/ plant	root length (cm)
IAA	88.0 a	2.9 a	1.1 a
NAA	24.4 c	1.0 b	0.8 ab
IBA	61.3 b	3.6 a	0.8 ab
2,4-D	0 d	0 b	0 b

[z]Mean separation by Fisher's protected LSD (P=0.05).

[y]Data percentage were arcsin-transformed before analysis. Untransformed means are presented.

CHAPTER 3

PLANT REGENERATION FROM SOMATIC TISSUE OF *RHODODENDRON laetum* x *aurigeranum*

A. Introduction

The majority of tissue culture studies on *Rhododendron* have focused on micropropagation by means of stimulating axillary shoot growth (Anderson, 1984; Fordham et al. 1982; McCown and Lloyd 1983; Blazich and Acedo 1988). This type of shoot multiplication has high genetic stability since somaclonal variation is minimized. However, this type of propagation has some limitations, such as lower rates of multiplication and in some species, for example *R. laetum* x *aurigeranum*, cannot be successfully applied (Iapichino et al. 1990).

In vitro propagation by adventitious shoots from somatic tissue provides a more efficient propagation method. Adventitious shoot formation from somatic tissue, with no or minimal callusing has been shown to be a useful means of mass propagation (Trevor and Patel, 1984). Theoretically, organogenesis can be induced from any somatic tissue and thus it does not require meristematic tissue, such as shoot tip explants. Furthermore, the development of an efficient shoot regeneration system from somatic tissues has been a prerequisite for *Agrobacterium*-

mediated transformations in dicotyledonous plant species.

There are a number of reports concerning plant regeneration from somatic tissues of _Rhododendron_. McCown and Lloyd (1983) have observed occasional production of adventitious shoots at the basal end of established shoot tip cultures in different _Rhododendron_ cultivars. Adventitious shoots were recorded by Fordham et al., (1982) on the stem and leaf surfaces of Exbury azalea shoot tips. Harbage and Stimart (1987) induced adventitious shoots on callus obtained from shoot tip cultures. Moreover, adventitious shoots have been regenerated from callus using ovary cultures from _R_._catawbiense_ (Meyer, 1982) and _R_. _prynophyllum_ (Day et al.1987). Shoot regeneration from the petiole end of _R_. 'PJM' hybrid leaves has been reported by Imel and Preece (1988). However, the potential of adventitious shoot regeneration for either micropropagation or for the development of genetic engineering methodology has not been explored.

The objectives of this study were: 1) to explore the feasibility of propagating _R._ _laetum_ _x_ _aurigeranum_ by adventitious shoot formation and 2) to improve the efficiency of plant regeneration from somatic tissues of _R_._laetum_ _x_ _aurigeranum_.

B. Materials and Methods

Plant material.

Stock plants of R. laetum x aurigeranum were grown in 22 cm. polyethylene pots containing a mixture of perlite, peat and soil (v/v/v=1:1:1). Greenhouse temperatures ranged from 26 to 32 C. Surface sterilization of the explants from greenhouse-plants was accomplished with the procedure described previously (Iapichino et al. 1990). Shoots were cultured on Anderson's medium (Anderson, 1984) containing 30 g/l sucrose, 100 mg/l inositol, 80 mg/l adenine sulphate, 8 g/l Difco Bacto Agar, 4 mg/l IAA and 15 mg/l 2iP for three months. Cultures were maintained under a 16 hour photoperiod, 50 u m^{-2} s^{-2} in Magenta GA 7 vessels (50 ml. medium per vessel). Experiments were performed using explants from greenhouse-grown plants and from axillary shoots proliferated in culture.

Experiment I

Two types of explants were used for these studies. The first type was from the sterilized shoot tips. The second type was from axillary shoots proliferated in culture. In both types the following material were used: shoot tip, single node, internodal section, whole leaf and leaf sections (strips).

the medium. Effort was made to ensure that the basal end of the shoot touching the medium surface was free of axillary buds. This was essential for distinguishing axillary buds from adventitious shoot proliferation on somatic cells.

2) Single nodes (3 cm. in length) bearing a fully expanded leaf were placed vertically on the medium. The basal portion (internodal portion) of the explant was in contact with the medium. The axillary bud was well above the medium surface.

3) Internodal sections (2 cm. in length) were placed vertically on the medium.

4) Detached leaves (2 mm. in length) were placed vertically on the medium. The petiole end was in contact with the medium.

5) Leaf blades were sliced perpendicular to the main vein; the resulting leaf strips (15 mm. in length and 5 mm. in width) were placed vertically on the medium.

Unless otherwise specified Anderson's medium (Anderson, 1984) containing 30 g/l sucrose, 100 mg/l inositol, 8 g/l agar and 80 mg/l adenine sulphate was used. IAA at 4 mg/l and 2iP at 15 mg/l were added to the medium prior to autoclaving. Ten ml of medium were dispensed into each 25 x 150 mm test tube and autoclaved at 121 C for 20 minutes. Five explants were used from each tissue type and from each plant material (greenhouse-grown plants and <u>in</u>

vitro cultured explants). A single explant was transferred into each test tube. The number of usable shoots (> 2mm.) was recorded after 6 months culture. The experiment was repeated twice.

Experiment II

In the second experiment three different types of explants were excised from the adventitious shoot culture: whole leaves with petiole attached, leaf strips (3 mm. x 3 mm.) and leaf sections (the top half leaf). Whole leaves were 10 mm. in length and 5 mm. in width. In order to obtain leaf strips of 3 mm. in length and 3 mm. in width, leaves were sliced perpendicular to the main vein. The bottom portion of the leaves, the petiole end, was removed to form leaf sections 5 mm. in length and 3 mm. in width. Explants were placed horizontally with the abaxial surface in contact with the medium.

Anderson's medium (Anderson, 1984) containing various combinations of IAA (0 to 6 mg/l) and 2iP (10 to 30 mg/10) concentrations was employed to determine the optimal growth regulator combination for inducing shoot regeneration. Growth regulators were added to the media prior to autoclaving. Ten ml of medium were dispensed into each 60 x 15 mm. petri dish. A completely randomized design was used. Each treatment consisted of 5 replications. A replicate consisted of a petri dish containing 4 explants. The percentage of shoots produced

was subjected to arcsin transformation prior to statistical analysis. The tests were evaluated for regeneration response by counting shoots with a minimum length of 2 mm (usable shoots).

C. Results

Shoot regeneration from selected explant tissue types

After 6 months in culture, shoot tip explants collected from greenhouse-grown plants produced only adventitious roots at the basal end. In contrast, shoot tips from *in vitro* proliferated shoots developed green callus at the basal end. This callus first turned brown and later produced adventitious shoots (Fig. 3.1A.). Shoots appeared to be seedling-like, with small leaves. An average of 8 shoots per explant was observed. Percent regeneration was about 60% (Table 3.1.).

Single nodes from greenhouse-grown plants showed tissue browning followed by necrosis, whereas single nodes from *in vitro* proliferated shoots developed an organogenic callus after six months in culture. Seventy percent regeneration occurred and an average of 12 shoots per explant was recorded.

The cultures of whole leaves derived from greenhouse-grown plants and from *in vitro* culture produced callus. This callus turned brown and eventually died. Similar

results were observed with leaf and internodal sections.

<u>Shoot regeneration from whole leaves, leaf strips and leaf sections</u>.

The lack of plant regeneration from leaf explants in the last experiments caused us to wonder if the plant growth regulator concentrations we used were not effective in inducing adventitious shoot regeneration. Therefore a further experiment was established using three types of explants exposed to various combinations of IAA and 2iP concentrations.

1) Whole leaves.

After 3 months of culture regeneration response was observed in the majority of the treatments. However, the frequency of regeneration did not exceed 30 % in all the treatments tested. Green primordia appeared at the basal end of the petiole and 2 months later these primordia differentiated into shoots (Figure 3.1B.). Occasionally the primordia produced numerous leaflets, resembling "moss" in appearance. However, a few shoots emerged subsequently from the rosettes of leaves. In those explants where regeneration did not occur either only callus formation or browning of the tissue explants followed by death of the explants was observed. IAA concentration of 6 mg/l induced the highest rate of callus formation. The callus turned brown and subsequently died. The highest

regeneration frequency (30%) occurred at 2 mg/l IAA and 20 mg/l 2iP (Table 3.2.). Increasing IAA and 2iP, respectively up to 6 and 30 mg/l, reduced regeneration and produced poor shoot growth. The number of shoots per leaf explant was low (less than 1 shoot per leaf) in all treatments tested.

2) Leaf strips

After 2 months, callus was observed in all treatments at both wound sites on the leaf strips. This callus first appeared green in color but had a very slow growth rate. Subsequently, the tissue turned brown and did not grow further. After 6 months, green primordia and later adventitious shoots appeared on the callus surface in some of the treatments. The highest shoot regeneration percentage was observed from explants exposed to 6 mg/l IAA and 20 mg/l 2iP (55%) (Table 3.3. and Fig. 3.1C.). The adventitious shoots appeared seedling-like with small leaves. Regeneration frequencies of 5 % occurred in treatments containing IAA at 2 mg/l and 2iP at either 20 or 30 mg/l. Equal rates of shoot regeneration occurred at either 4 mg/l IAA and 10 mg/l 2iP or 6 mg/l IAA and 30 mg/l 2iP. The number of shoots produced was significantly influenced by the various concentrations of growth regulators (Table 3.4.). About 7 shoots per callus were induced in explants cultured on medium supplemented with 6 mg/l IAA and 20 mg/ 2iP.

3) Leaf sections

Table 3.3. depicts the responses obtained from leaf sections exposed to the different media tested. Shoot primordia were observed about two months after cultures were established. The highest percentage of primordia was recorded in the medium supplemented with 4 mg/l IAA and 30 mg/l 2iP (50%). IAA was necessary for primordia development. In the treatments where morphogenic responses were observed, primordia formed at the cut edges of the leaf sections with minimum amounts of callus (Fig.3.1D.)

D. Discussion

Previous experiments (Iapichino et al. 1990) indicated that shoot tip explants from greenhouse-grown plants, cultured horizontally on Anderson's medium supplemented with 4 mg/l IAA and 15 mg/l 2iP, produced a limited amount of axillary shoots. Those explants eventually produced callus. Adventitious shoots were produced from callus developed at the cut surfaces of explants. In the present study neither axillary nor adventitious shoot regeneration occurred from shoot tips placed vertically in test tubes ; only root formation was observed. Similarly, we obtained adventitious shoots at the petiole basal end of leaves placed horizontally, whereas, leaves placed vertically did not show any regeneration response.

These results could be due either to the different placement of explants (vertical vs horizontal) on the medium and/or to the different container sizes that were used during the experiments.

Horizontal placement of explants on medium is reported to increase axillary shoot proliferation in lilac (Hildebrandt and Harney, 1983), pears (Lane, 1979) and French tarragon (Mackay and Kitto, 1988) compared to vertical placement. Horizontal orientation is believed to result in greater uptake of medium constituents due to increased contact with the medium (Mackay and Kitto, 1988).

Container size has also been shown to affect shoot proliferation (Monette, 1983; Start and Cumming, 1976). This is thought to be due to the ratio between the volume of air and medium available to the explants. The volume ratio between medium and air contained in 20 x 100 mm. test tubes is considerably less than that contained in Magenta GA 7 vessels used in a previous experiment (Iapichino et al. 1990).

In this experiment we also observed that shoot tip and single node explants from greenhouse-grown plants did not regenerate shoots, whereas the respective explants from <u>in vitro</u> culture produced adventitious shoots. This phenomenon could be due the acquisition of competence of the tissues for shoot regeneration after exposure to <u>in vitro</u> conditions. Shoot rejuvenation after serial

subcultures has been reported as a possible cause of enhanced shoot multiplication in birch (McCown and Amos, 1978).

A low regeneration frequency was obtained when whole leaves of _R. laetum x aurigeranum_ were used as explant sources. Fordham et al. (1982) reported similar findings with Exbury azalea. Regeneration from whole leaves was sporadic, required a long period of time and was confined to the wound site of the petiole surface.

Shoot regeneration was accomplished from leaf strips and leaf sections of _R. laetum x aurigeranum_ . When comparing the adventitious shoot regeneration from leaf strips with whole leaves, the regeneration is much more efficient in the leaf strips. The reason for such a different response is not known. A long intermediary callus phase was required using leaf strips as explant sources. Conversely, our preliminary results concerning shoot regeneration from leaf sections showed that shoot primordia differentiation can be accomplished in a shorter time than that required from leaf strip explants and with a limited amount of callus formation. It is known that adventitious shoot formation from leaf section explants can involve different amounts of callus formation. Callus proliferation followed by adventitious shoot regeneration was observed on _Solanum tuberosum_ leaf discs by Weeb et al. (1983). However, shoots regeneration without callus formation was observed

in blueberry leaf explants (Callow et al.1989). Of several strawberry cultivars tested, only two showed direct regeneration without a callus phase, whereas the other genotypes formed adventitious shoots via intermediary callus (Narender et al. 1989). This suggests that, regardless of whether callus is formed or not, prior to adventitious shoot regeneration, the response is genotypic.

The duration of exposure to the medium, during the regeneration process, may be as important as the composition of the medium for the acquisition of competence for shoot induction (Christianson and Warnick, 1988). Shoot regeneration from leaf sections occurred in _R. laetum x aurigeranum_ after 6-7 months culture. The same time was required to induce organogenesis from callus formed at the cut surfaces of shoot tip explants of the same _Vireya_ hybrid (Iapichino et al 1990). Wolter (1968) reported that aspen (_Populus tremuloides_) callus displayed shoot regeneration when left on the same medium for several months. Aging and hormonal balance changes that occurred in the tissue with time were suggested as possible explanations. However, Eun and Minocha (1986) were able to induce organogenic callus in only one month in the same species by manipulating medium and growth regulators. This suggests that by altering medium composition and growth regulator concentrations, the efficiency of plant regeneration from somatic tissues of _R. laetum x_

aurigeranum could be further improved.

Fig. 3.1. A - Adventitious shoot proliferation at the basal end of axillary shoot (arrow head indicates the original shoot), originated from *in vitro* grown cultures of *R. laetum x aurigeranum*. Shoot explant was cultured on Anderson's medium supplemented with 4 mg/l IAA and 15 mg/l 2iP for 6 months. B - Adventitious shoot induced at the petiole end of leaf explants after 5 months culture on Anderson's medium supplemented with 2 mg/l IAA and 20 mg/l 2iP. C - Callus and adventitious shoot formation on leaf strips of *R. laetum x aurigeranum* after 6 months culture on Anderson's medium supplemented with 6 mg/l IAA and 20 mg/l 2iP. D - Shoots and shoot primordia formation on leaf sections of *R.laetum x aurigeranum* after 2 months culture on Anderson's medium supplemented with 4 mg/l IAA and 30 mg/l 2iP.

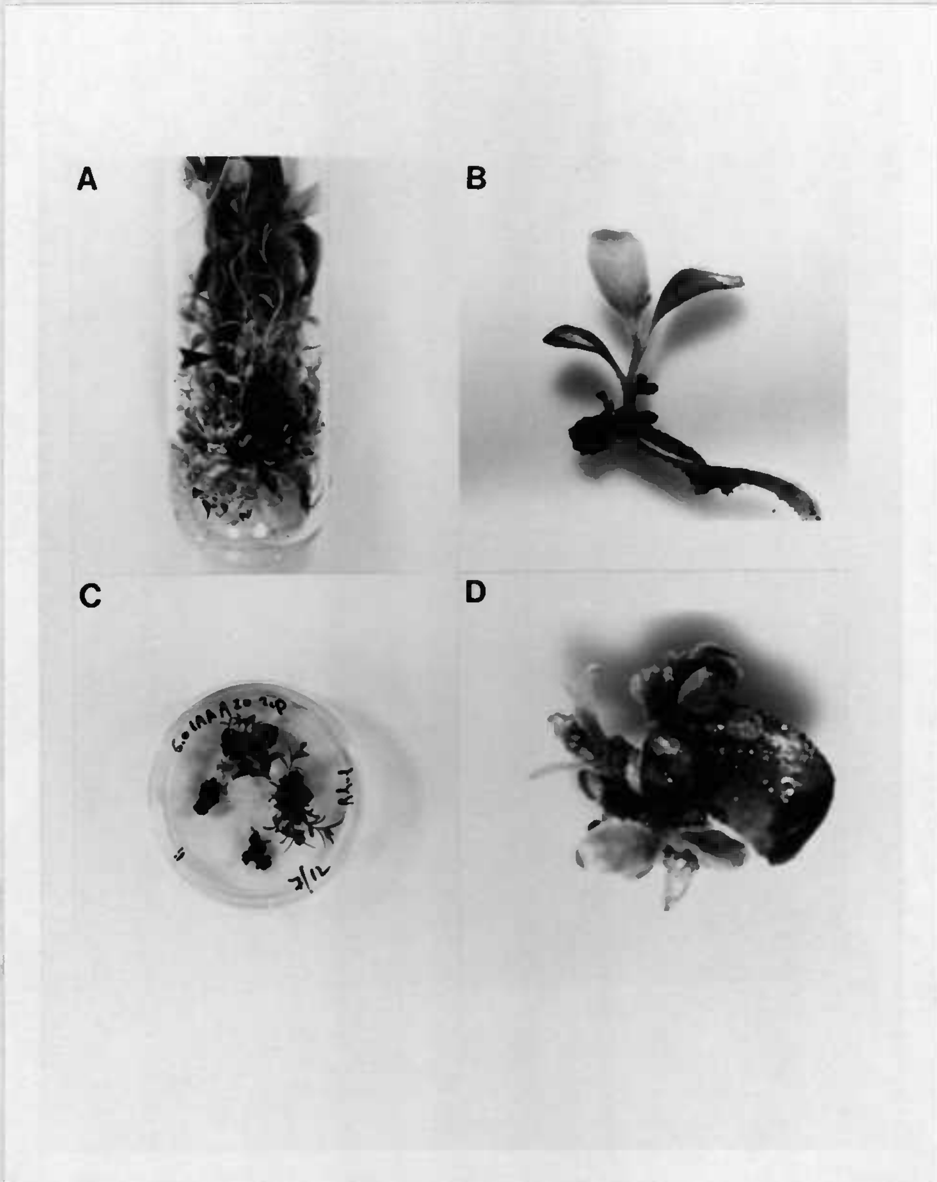

Figure 3.1.

Table 3.1. Response of different explant types of *R. laetum x aurigeranum* to Anderson's medium supplemented with 4 mg/l IAA and 15 mg/l 2iP.

Plant material	Type of explant	Regeneration %	No. shoots/ explant
In vitro cultured shoot	Shoot tip	60	7.6 ±10.3
	Single node	70	11.8 ±9.1
	Whole leaf	0	0
	Leaf section	0	0
Greenhouse grown plant	Shoot tip	0	0
	Single node	0	0
	Whole leaf	0	0
	Leaf section	0	0

Table 3.2. The response of whole leaf explants of R. laetum x aurigeranum to various combinations of IAA and 2iP after 5 months in culture.

Growth regulators (mg/l)		Regeneration %	No. Shoots/ explant	*callusing %
IAA	2iP			
0	10	15	0.1	5
"	15	15	0.4	5
"	20	20	0.6	20
"	30	15	0.3	20
2	10	15	0.2	40
"	15	10	0.1	20
"	20	30	0.5	35
"	30	20	0.6	5
4	10	10	0.1	35
"	15	0	0	40
"	20	5	0.05	30
"	30	25	0.45	40
6	10	25	0.5	30
"	15	15	0.25	70
"	20	20	0.3	80
"	30	5	0.05	25
LSD 0.05		19.3	0.2	29.2

*Percentage data were arcsin transformed before analysis. Untransformed means are presented.

Table 3.3. Effect of various combinations of IAA and 2iP concentrations on shoot regeneration from leaf strips of _R. laetum x aurigeranum_ after 6 months in culture.

2iP (mg/l)	IAA (mg/l)		
	2	4	6
	Regeneration %[y]		
10	0 b	5 b	0 b[z]
15	0 b	0 b	0 b
20	5 b	0 b	55 a
30	5 b	0 b	5 b

[z]Mean separation by Fisher's protected LSD (P=0.01).

[y]Percentage data were arcsin transformed before analysis. Untransformed means are presented.

Table 3.4. Effect of various combinations of IAA and 2iP concentration on shoot regeneration from leaf strips of *R. laetum* x *aurigeranum*. (time of culture 6 months).

	IAA (mg/l)		
2ip (mg/l)	2	4	6
	No. shoots/explant		
10	0 b	0.4 b	0 b[z]
15	0 b	0 b	0 b
20	0.05 b	0 b	7.2 a
30	0.1 b	0 b	0.3 b

[z]Mean separation by Fisher's protected LSD (P=0.01).

Table 3.5. The response of leaf section explants of R.laetum x aurigeranum to various combinations of IAA and 2iP after 2 months in culture.

Growth regulators (mg/l) IAA	2ip	Regeneration %[z]	Callusing %
0	10	0	30
"	15	0	45
"	20	0	20
"	30	0	20
2	10	10	60
"	15	20	35
"	20	20	40
"	30	10	45
4	10	20	40
"	15	10	50
"	20	25	30
"	30	50	35
6	10	10	25
"	15	5	30
"	20	5	50
"	30	25	20
LSD 0.05		19.4	NS

[z]Percentage data were arcsin transformed before analysis. Untransformed means are presented

CHAPTER 4

ADVENTITIOUS SHOOT REGENERATION FROM LEAF EXPLANTS OF RHODODENDRON

A. Introduction

Adventitious shoot formation from somatic tissue by *in vitro* plant regeneration has important applications for rapid mass propagation and for genetic engineering. Protocols have been developed for genetic transformation of many herbaceous species (Horsch et al., 1985; Shermann and Bevan, 1988; McCormick, et al. 1986). Alteration of pigment biosynthesis to generate new flower color patterns was obtained in petunia by using *Agrobacterium* mediated transformation (Van der Krol et al. 1988). Due to the complexity of the breeding process in *Rhododendron* spp., the availability of similar approaches, i.e. genetic engineering, to alter flower color traits would have tremendous commercial implications. However, a critical step in genetic engineering is the ability to regenerate plants from transformed cells. The development of an efficient regeneration system from *Rhododendron* leaf explants is the first step toward the exploration of gene transfer techniques in *Rhododendron* spp.

There are a number of studies concerning shoot

regeneration from Rhododendron leaf explants: adventitious buds have been observed by Fordham et al. (1982) on the abaxial and adaxial surfaces of Exbury azalea leaves attached to shoot tip explants; Imel and Preece (1988) reported successful shoot regeneration at the petiole end of Rhododendron 'P.J.M' leaves. In both cases, whole leaves were used as explants. No study has documented direct shoot regeneration from Rhododendron leaf segments. Recently, we were able to regenerate plants from leaf segments of R laetum x aurigearanum (Iapichino et al. 1990). The successful regeneration in that species led us to explore the possibility of plant regeneration from leaf tissues of commercial Rhododendron cultivars. The objective of this study was to develop a shoot regeneration protocol from leaf segments of Rhododendron cultivars.

B. Materials and Methods

Shoot cultures of 10 white-flowered Rhododendron cultivars were obtained from Briggs Nursery, Inc. The cultivars used in this study were: 'Ivory Coast', 'Princess Yaku', 'Joe Paterno', 'Lodestar', 'R. catawbiense album', 'Lodery King George', 'Molly Fordham', 'Anna H. Hall', 'Cunninghams', 'Dexter's Spice'. Shoots were subcultured every 4 weeks on Anderson's medium (Anderson, 1984) supplemented with 5 mg/ 2iP. Cultures were maintained under a 16 hour photoperiod and 50 um^{-2} s^{-2} light intensity in Magenta GA 7 vessels.

Leaves from each of the cultivars were excised and sliced perpendicularly to the main vein to form leaf strips of 5 mm. in length and 3 mm. in width. Leaf strips were placed horizontally with the abaxial surface in contact with the medium.

The leaf strips were cultured in Anderson's medium (Anderson, 1984), containing 30 g/l sucrose, 100 mg/l inositol, 80 mg/l adenine sulphate and 8 g/l Difco Bacto Agar. A factorial experiment with various combinations of IAA (0 to 10 mg/l) and 2iP (1 to 15 mg/l) concentrations was employed to determine the optimal growth regulator concentration for inducing organogenesis from leaf fragments in each cultivar. Growth regulators were added to the medium prior to autoclaving at 121 C for 20 minutes. Ten ml. of medium were dispensed into each 60 x 15 mm. petri dish. A completely randomized design was used for each cultivar tested. The experiment was repeated 2-10 times depending on the availability of plant material. A replicate consisted of a petri dish containing 4 explants. Data were subjected to arcsin transformation prior to statistical analysis. Percent regeneration was defined as the number of explants developing one or more usable shoots (> 2 mm. in length).

C. Results and Discussion

Two to three weeks after culturing, 30 % of the explants turned brown. The remaining explants were green and showed swelling and callus formation at the wounded edges (Fig.4. 1A.). Callus formation was minimal and shoot primordia were visible, after 4 weeks of culture, on the surface of these explants (Fig.4.1B.). Buds developed completely after 2 months in culture. Subsequently, shoot primordia differentiated on the area in contact with the medium and eventually on the entire explant (Fig.4.1C.). Shoots continued to grow and multiplication was enhanced by subculturing clusters of shoots into fresh Anderson's medium supplemented with 5 mg/l 2iP (Fig.4.1D.).

Genotypic differences were found in the _in vitro_ shoot regeneration ability of _Rhododendron_ leaf strips (Table 4.1.). Among the 10 cultivars tested only three showed reproducible plant regeneration response (Table 1). No regeneration response was recorded for the other cultivars.

The highest shoot regeneration percent (22.5 %) was obtained from 'Ivory Coast' leaf explants cultured on Anderson's medium supplemented with 5 mg/l IAA and 5 mg/l 2iP (Table 1). IAA and 2iP concentrations higher or lower than 5 mg/l reduced shoot regeneration in this cultivar. Shoot regeneration (5%) occurred in 'Joe Paterno' leaf strips cultured on media supplemented with 2 or 5 mg/l IAA and 15 mg/l 2iP. In 'Lodestar' a regeneration frequency of

5 % was observed on medium supplemented with 5 mg/l IAA and 15 mg/l 2iP as well as on medium with 10 mg/l IAA and 1 mg/l 2iP.

Adventitious shoot regeneration from leaf explants has been documented for other woody species: blueberry (Billing et al. 1988; Dweikat and Lyrene, 1988; Callow et al., 1989), pomegranate (Omura et. al 1987), aspen (Noh and Minocha, 1986) and fig (Jona and Gribaudo, 1987). The number of plants regenerated from somatic woody plant tissue is increasing. Our study demonstrates that adventitious shoot regeneration from leaf explants of *Rhododendron* is possible. However, regeneration responses vary among cultivars and is also greatly affected by growth regulator concentration. 'Ivory Coast' gave the highest regeneration response among the cultivars tested (5 shoots /explant). To the best of our knowledge, this is the first report concerning plant regeneration from leaf fragments of commercial *Rhododendron* cultivars. We are currently exploring the possibility of transforming the regenerable *Rhododendron* cultivars by *Agrobacterium* mediated transformation.

Fig. 4.1. The response of *Rhododendron* 'Ivory Coast'leaf explants cultured on Anderson's medium supplemented with 5 mg/l IAA and and 5 mg/l 2iP. A - Swelling and callus formation at the wound edges of leaf sections after 3 weeks in culture. B - Shoot primordia formation after 4 weeks in culture. C - Cluster of adventitious shoots. D - Adventitious shoot enhancement after 2 months in culture.

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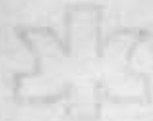

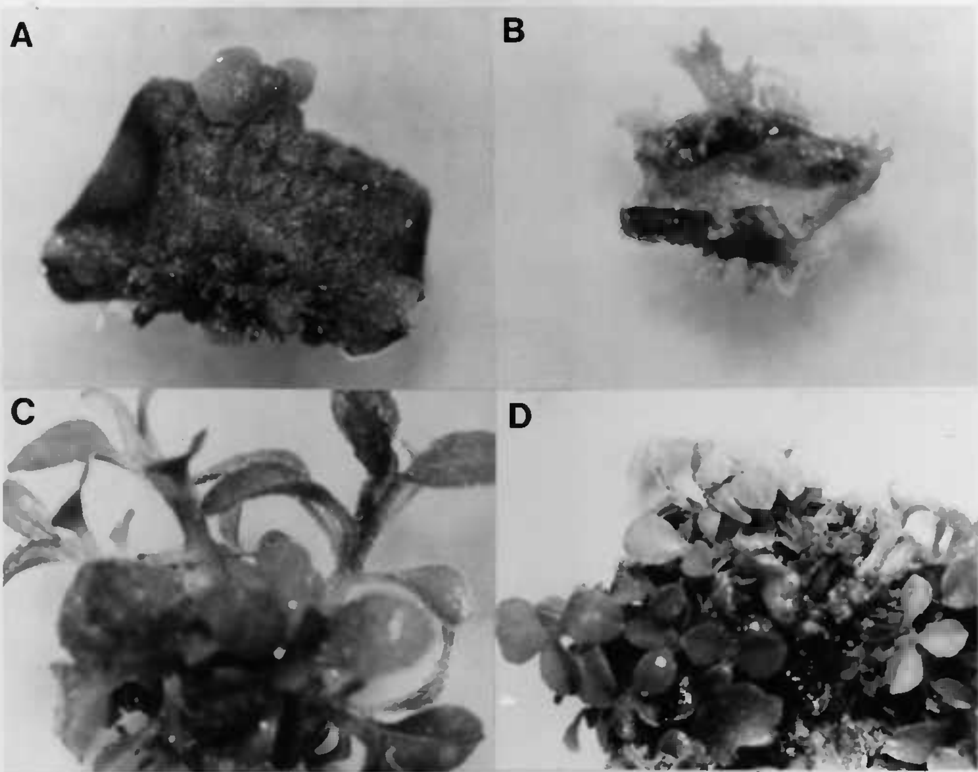

Figure 4.1.

Table 4.1. Effect of various combinations of IAA and 2iP concentrations on shoot regeneration from leaf strips of 3 Rhododendron cultivars.

Growth regulator (mg/l)		Cultivar		
IAA	2iP	Ivory Coast	Joe Paterno	Lodestar
		Regeneration (%)		
0	1	2.5	0	0
"	5	2.5	0	0
"	15	0	0	0
1	1	5.0	0	0
"	5	0	0	0
"	15	0	0	0
2	1	2.5	0	0
"	5	0	0	0
"	15	5.0	5.0	0
5	1	0	0	0
"	5	22.5	5.0	0
"	15	2.5	0	5.0
10	1	2.5	0	5.0
"	5	7.5	0	0
"	15	7.5 (10)*	0 (5)	0 (2)

*The number in parenthesis indicates the number of replications.

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