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Propagation of Wild Rose for Reclamation by Breaking Seed Dormancy, Rooting Stem Cuttings and *In Vitro* Techniques

BY

DEBORAH ANNETTE BIGELOW

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled MASS PROPAGATION OF WILD ROSE FOR RECLAMATION BY BREAKING SEED DORMANCY, ROOTING STEM CUTTINGS AND *IN VITRO* TECHNIQUES submitted by DEBORAH ANNETTE BIGELOW in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in BOTANY.

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Abstract

The potential for propagation of wild rose by breaking seed dormancy, rooting stem cuttings and callus production through *in vitro* techniques was investigated. Mature wild rose achenes were separated into viable and non-viable categories via specific gravity with greater success than immature achenes, and therefore were used in further experimentation. Dual stratification (25°C ± 2°C / 5°C ± 2°C) provided a means of breaking dormancy. Germination of achenes exposed to warm conditions followed by cool conditions was less than 25%, while achenes exposed to the reverse situation resulted in less than 10% germination. *In vitro* techniques using cut and whole achenes was not successful. Complete contamination of the cultures resulted in failure of the achenes to germinate. The effect of cutting the achenes in half transversely, acid scarification and mechanical abrasion of the seed coat on breaking dormancy and enhancing germination was also studied. Cutting the achenes was the only treatment that produced substantial germination, resulting in 82% cumulative germination. The other treatments resulted in 0 to 20% germination of achenes.

Rooting of stem cuttings provided an effective means of establishment, but success in rooting varied with the supply of rooting hormones (0, 0.1, 0.3, and 0.8% indolebutyric acid (IBA)), the type of rooting media (sand, loam and metro mix), the seasonal status of stem cuttings (hardwood, softwood and semi-hardwood) and stem defoliation. The highest root production occurred in softwood stem cuttings treated with 0.8% IBA. Loam and metro mix were the most effective rooting media as evidenced by the highest number of roots produced. Defoliation of stem cuttings decreased root produced by almost 2.5 times that of foliated cuttings.

Despite progress in developing procedures for in vitro propagation of wild rose, successful regeneration of whole plants from callus cultures was not achieved. The effect of naphthaleneacetic acid (NAA) (0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mgL^{-1}) and benzylaminopurine (BAP) (0, 0.25, 1.00, 2.00, 3.00 and 4.00 mgL^{-1}) on the induction of callus on leaf discs of wild rose were studied. Callus growth increased with a corresponding increase in NAA. In contrast, there was no consistent growth response to BAP, although both growth regulators were required in the media to promote callus induction. The effect of NAA (0.4, 0.6, 0.8 and 1.0 mgL^{-1}) and BAP (2.00, 3.00, 4.00, 6.0 and 9.0 mgL^{-1}) on the continual growth of callus during sub culturing was also studied as growth increased with each subculture. The general trend illustrated a reduction in growth with an increase in NAA. From the results, only general recommendations can be made. For callus induction, NAA concentrations greater than 0.6 mgL^{-1} and BAP concentrations greater than 0.25 mgL^{-1} can be used, while for callus growth, NAA concentrations less than 0.6 mgL^{-1} and BAP concentrations less than 4.0 mgL^{-1} were found to be most effective.

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1. General Introduction

The genus *Rosa* in Western Canada is taxonomically complex due to within species variation and hybridization between species where ranges overlap. Two of the species native to Alberta, *Rosa acicularis* (prickly rose) and *Rosa woodsii* (common wild rose), are commonly misidentified as a result of similar morphology and distribution. Both species are long lived perennials that occcur as bushy shrubs 0.5 - 2.5 m in height (Hardy BBT Ltd, 1989). The fruit of roses are achenes, which are borne within fleshy hips. Both species are also adapted to a wide range of soils except those that are very wet or acidic (Haeussler *et al.*, 1990). They also thrive on moderately fertile, well-drained clay loam, sandy loam or sandy soils (Hardy BBT Ltd, 1989) which increases their potential use in reclamation.

In Alberta, revegetation trials on disturbed areas have lead to the current status of wild rose (*R. acicularis* and *R. woodsii*) in reclamation. Wild rose is common on disturbed sites and has been reported as a pioneer plant as a result of the rapid cover rate and ability to invade gravel and silt bars, and areas where mineral soils are exposed (Hardy BBT Ltd, 1989). As a result of the rapid cover rate and the ability to spread by rhizomes, wild rose is capable of contributing to erosion control and soil building. Among the natural assests of wild rose for reclamation are, tolerances to wide ranges of climate, moisture, light and nutrients, which contribute to high survival rates throughout Alberta (Hardy BBT Ltd, 1989). Both species are also beneficial to birds, mammals and ungulates as a year round source of food and shelter (Haeussler *et al.*, 1990).

Once established, wild rose has the ability to spread by rhizomes and buried root crowns or to germinate from on-site or transported seeds. The first seed production occurs when the plant is 2 - 5 years old with seed dormancy lasting 12 - 15 months

(Haeussler *et al.*, 1990). Although wild rose has proven to be a suitable species for reclamation of diverse environments, its use has encountered setbacks. The industry requires large quantities of vigorous plants adapted for use on specific disturbed sites at low cost. Present techniques for propagation of *Rosa* focus on horticultural species with limited protocols for wild rose propagation. Therefore, the overall objective of this research was to develop techniques to propagate wild rose on a large scale for the purpose of encouraging the use of this species in reclamation. Traditionally, horticultural roses have been propagated by grafting, budding or rootstocks which are labor intensive and time consuming methods (Skirvin *et al.*, 1990). Alternate systems for rose propagation include seed propagation, cutting propagation and tissue culture or micropropagation.

Propagation of roses by seed is often unpredictable and inconsistent. Seeds of angiosperms are the culmination of the double fertilization of the ovule within the ovary (Murray, 1984). In wild rose, the seeds are found within fleshy fruits or hips. The structure of a rosaceous seed consists of an embryo, endosperm and testa or seed coat (Mayer and Poljakoff-Mayber, 1982). The embryo is the result of the fusion of the nuclei of the male and female gametes and consists of a radicle, plumule, cotyledons and hypocotyl (Mayer and Poljakoff-Mayber, 1982). In rosaceous species, the embryo has two cotyledons and almost completely fills the seed. The testa normally develops from the integuments of the ovule (Mayer and Poljakoff-Mayber, 1982) and provides a barrier between the embryo and the immediate environment. In wild rose, the embryo is within a thin testa and surrounded by a thick sutured pericarp (King *et al.*, 1983). Unfortunately this complicates germination, as both the testa and the pericarp must be broken down to allow imbibition of water and eventual germination (Skirvin *et al.*, 1990). Therefore, propagation of wild rose by seed often results in wide germination differences as a result of embryo dormancy (Dirr and Haeussler Jr., 1987), coat-imposed dormancy (Villiers, 1972) or both. Harper (1959) recognized three general types of dormancy; innate,

induced and enforced. Innate or primary dormancy prevents germination during development and maturation of the seed. Induced or secondary dormancy is the persistence of the dormant condition following the return of the seed to favorable conditions for germination (Harper, 1959). Seeds that are subjected to enforced or imposed dormancy, germinate immediately upon removal of environmental limitations (Harper, 1959).

Despite the type of dormancy exhibited by wild rose achenes, high rates of germination have been achieved with combinations of warm and cold stratification. King and coworkers (1983) obtained greater than 50% germination with 60 days warm and 120 days cold stratification for seeds that were immature, but less than 35% germination for ripe seeds with the same stratification. Therefore, collection time of achenes also influences germination, as a higher level of dormancy is expected to occur due to the accumulation of chemical inhibitors as the seeds ripen (Dirr and Haeussler Jr., 1987).

Roses can also be propagated by cuttings, which provide a method to maintain specific characteristics of the stock plant. Cuttings can be taken from any vegetative portion of a plant such as leaves, stems and roots (Dirr and Haeussler Jr., 1987). Leaf cuttings are generally not suitable for propagation of woody plants, but root and stem cuttings have the potential for propagation of rose species. Although root cuttings have a high propensity to produce shoots, this method is inappropriate for propagating large numbers of plants, because of the intense labor demand. Stem cuttings are the preferred method of propagation and can be divided into softwood, semi-hardwood and hardwood (Dirr and Haeussler Jr., 1987) based on the time of year and status of the stock plant. Hermesh and Cole (1983) achieved success rates for rooting rose from 30 - 100% for hardwood cuttings and from 5 - 63% for softwood cuttings. Various parameters

involving rooting hormones such as auxins, and rooting media have been tested in the past with a variety of results (Hermesh and Cole, 1983; Chong and Daigneault, 1990).

Wild rose can also be propagated by micropropagation through *in vitro* techniques. Micropropagation is a method that has potential to clone millions of identical plants from a single individual (Donnelly, 1990). The three stages of micropropagation include establishment, multiplication and preparation for re-establishment (Murashige, 1986). The objective of stage one is the establishment of a sterile culture. Factors that affect establishment include explant source, prevention of contamination, proper culture medium and appropriate culture conditions. The objective of stage two is the rapid multiplication of callus or organs that will eventually give rise to a complete plant. The function of stage three is the preparation of plantlets for establishment in a natural environment. Stage three involves the variation of growth regulators to enhance shoot production and initiate rooting (Murashige, 1986).

The first major steps in developing a micropropagation technique were taken in 1922 by Robbins and Kottee (Thomas and Davey, 1975). Intact meristems of grass seedlings were excised from root tips which grew to form small root systems. Unfortunately growth rapidly declined and eventually ceased in the culture system. However, other culture studies quickly followed. Gautheret (1969) succeeded in promoting development of wound tissue on callus from species of *Salix* and other woody plants. His success was influenced by the discovery of the naturally occurring auxin, indole-3-acetic acid (IAA), by Thimann and Went in 1934. This discovery lead to the synthesis of other auxin compounds capable of regulating plant cell elongation such as naphthaleneacetic acid (NAA). Skoog and Miller (1957) discovered a synthetic cytokinin, kinetin, when attempting to culture a wound callus formed on *Nicotiana tabacum.* Cytokinins are a class of naturally occurring plant growth hormones which are capable of

promoting active growth of tissue (Kaminek, 1992). Therefore, inclusion of a combination of auxin and cytokinin in the culture medium allows for more precise control of callus formation, and the initiation of roots and shoots from callus. As tissue culture occurs *in vitro*, or in isolation from the rest of the plant body, the system must be supplied with essential inorganic salts, organic factors such as vitamins, amino acids and a carbon source in addition to growth regulators (Thomas and Davey, 1975). These components, which are essential for growth, are provided through the culture medium. The basal medium supplemented with vitamins and growth regulators for tissue culture supports growth rates which are many orders of magnitude greater than in the greenhouse or field (Donnelly, 1990). Following shoot production, growth regulators can be altered to stimulate root development resulting in numerous plantlets (Hu and Wang, 1986). Once the root to shoot ratio is 1:2, the plantlets are acclimatized by maintaining a high humidity environment to prevent stress during transplant to the field or greenhouse (Donnelly, 1990).

Advantages of micropropagation, such as rapid clonal propagation of plants in a space-economical manner, allows propagators to respond quickly to market trends without concern for seasonality (Donnelly, 1990). Another advantage of this technique is the production of extremely uniform and high quality plant material that can exist free of soil or compost during the multiplication stage. This stage can be extended by subculturing the plantlets onto fresh medium which also facilitates the transport of propagules with minimal maintenance or damage to the product (Donnelly, 1990). Limitations to this technology deals with contamination problems, and the technical problems involved in propagating new species. Despite these limitations, micropropagation is now used internationally for cloning plants. In 1987 it was estimated that there were approximately 300 micropropagation businesses worldwide. Now there

are more than 250 laboratories in North America alone, each with a planned production capacity of 5 to 20 million plantlets (Donnelly, 1990).

Due to the lack of established methods for propagating large numbers of plants of wild rose in a condition suitable for out-planting, the objective of this research was to devise propagation techniques that would increase the availability of wild rose for reclamation projects. Specific objectives included (1) breaking seed dormancy and enhancing the germination of wild rose achenes, (2) enhancing rooting of stem cuttings by testing the effects of various rooting hormones, rooting media and time of cuttings and (3) assessing the influence of auxins and cytokinins on callus formation and organogenesis in an attempt to regenerate plantlets.

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2. Propagation of Wild Rose by Breaking Dormancy and Enhancing Germination in Achenes

2.1 Introduction

Wild rose first flowers and sets seed 2 - 5 years after germination (Haeussler *et al.*, 1990) with the hips containing the achenes remaining on the mother plant until late winter or early spring. While still attached, the embryo will eventually reach its maximum size followed by ripening and dehydration (Bryant, 1985). The dehydrated seed is quiescent with extremely low metabolic activity (Bryant, 1985). At this stage the seed is shed and is ready to germinate under appropriate conditions. However, many species, including wild rose, will not germinate at this time as they are not merely quiescent, but dormant (Bryant, 1985).

With the breaking of dormancy, three phases of development lead to germination. Germination begins with imbibition of water and culminates with the emergence of the radicle. Come and Thevenot (1982) have followed the progression of imbibition through the three stages of development in *Malus*. As *Malus* belongs to the *Rosaceae*, it is possible to assume a similar sequence of events for wild rose. Come and Thevenot (1982) observed a rapid initial absorption of water followed by a gradual slowing to establish a stable water content. Imbibition again increased leading to a lag in germination which coincides with the second phase of development, the activation phase. During this lag phase, the seed is preparing for germination as evidenced by changes in enzyme activity and metabolic pathways (Simon, 1984). The third and final stage of development is the growth phase which is characterized by radicle elongation and protrusion through the seed coat (Come and Thevenot, 1982), culminating in germination. As the radicle continues to elongate, the style of germination exhibited by wild rose is epigeal with the emergence

of the cotyledons as the first photosynthetic organs of the seedling (Mayer and Poljakoff-Mayber, 1982).

While long term dormancy is rare among cultivated plants, it is common among seeds of wild plants such as the native wild rose (Black, 1972). Dormancy of the wild rose achenes usually lasts 12 - 15 months, preventing germination until appropriate environmental conditions occur. Suitable temperatures, light and an adequate supply of water and oxygen must be available to the seed once dormancy is removed. As dormancy is a condition of nature which has evolved to ensure a seed's survival (Fordham, 1990), the natural environment that the seed is subjected to should be considered in the treatment to remove or prevent dormancy. There is no consistent agreement in the current literature as to the type of dormancy that occurs in wild rose. Bryant (1985) suggested that dormancy is either coat or embryo imposed, or possibly a combination of the two. Whatever type of dormancy is expressed by the seed, it is generally due to a variety of causes such as impermeability of the seed coat to water and gases, mechanical restraint to embryo development, immaturity of the embryo and the need for special requirements of light or temperature (Mayer and Poljakoff-Mayber, 1982). Therefore, removal of dormancy reflects the interaction of a number of ecological and physiological dormancy breaking cues. As a result, germination rates of the native wild rose tends to be low and unpredictable, hindering its use in reclamation. Development of techniques that consistently result in high germination percentages over a short period would be valuable in encouraging the use of native wild rose in revegetation projects.

There is little substantive information in the literature on the propagation of wild rose from seed, and attempts to break seed dormancy have produced inconsistent results. Even under controlled conditions, germination of wild rose is difficult. To some extent, stratification (King *et al.*, 1983), acid treatment (Roberts, 1979), scarification (Villiers,

1972; Smithberg and Gill, 1974) and *in vitro* techniques (Miller *et al.*, 1992) have been successful in enhancing germination of rose hybrids and other rosaceous species. However, wild rose achenes appear to be fairly insensitive to these treatments.

The practice of stratification by placing seeds in a moist, low temperature environment, usually 5°C (King *et al.*, 1983), is a common strategy in breaking dormancy. However, successful germination as a result of stratification is species-specific and may even vary within species depending on embryo maturity and previous environmental conditions (Come and Thevenot, 1982). Densmore and Zasada (1977) reported that the optimal environment for germination of Alaska *Rosa acicularis* achenes was a dual stratification of 118 days of warmth followed by 90 days of cold. Later studies by King *et al.* (1983) found maximum levels of germination in immature seeds with a shorter warm stratification period followed by a longer cold period, but under the same conditions, germination of ripe seeds was much lower. Other studies using acid treatment in combination with stratification to overcome seed dormancy of wild rose substantially reduced the time required for warm stratification (Roberts, 1979). Mechanical scarification of the seed coat has also been found to shorten germination time by weakening the physical barriers surrounding the embryo (Smithberg and Gill, 1974). However, others have encountered low germination rates when employing scarification techniques (Villiers, 1972) and therefore do not consider this method a beneficial pre-germination treatment.

A current method that has proven effective in overcoming the inhibition of germination is embryo culture using *in vitro* techniques. The ability to germinate seeds on semisolid sucrose-agar medium lacking hormones advances the traditional strategies for breaking dormancy. Arrillaga *et al.* (1992) and Miller *et al.* (1992) developed tissue culture protocols that increased germination percentage and decreased the lag time to

germination for several *Rosa* species. Comparative treatments of intact achenes and excised embryos have demonstrated higher germination rates in embryos that were removed from the restraints of the seed coat (Miller *et al.*, 1992).

The overall objective of the present study was to determine methods for breaking seed dormancy and enhancing germination of wild rose. Several treatments including (1) stratification, (2) acid scarification, (3) mechanical scarification, (4) cutting the achenes in half and (5) *in vitro* culture were compared to assess their potential for increasing germination percentage while simultaneously decreasing the lag time to germination. In addition, a modified form of the Weibull function was used to quantitatively describe cumulative germination of the most promising method to enhance germination.

2.2 Methods

Rose hips were collected from Rainbow Valley in Southwest Edmonton at two phases of ripeness, immature or "not fully ripe", and ripe. Immature hips were collected in late July and were yellowish-orange and firm in texture. Ripe hips were collected in early September and were red in color with a softer texture. After collection, seeds were removed from the hips and temporarily stored at 5°C. Prior to treatment, a viability test was performed on all seeds by submersing in distilled water on a magnetic stirrer for two minutes. Seeds were then left to stand until separation by specific gravity was complete. Achenes with high specific gravity sank and were presumed viable. Those with low specific gravity floated and were presumed nonviable. Viability tests of the two groups was confirmed by cutting through the seed coat and exposing the embryo to allow for visual assessment of viability. Embryos that were white in color with an endosperm that filled the seed were presumed to be viable, while embryos that were brown and shrunken from the testa were considered nonviable. Following the preliminary seed viability test,

putatively viable seeds were surface sterilized in 70% ethanol for 30s followed by soaking in 0.5% (w/v) sodium hypochlorite (bleach) for 15 minutes and then rinsed three times in distilled water in preparation for further experimentation.

In the first experiment, surface sterilized seeds were dual stratified at 5°C ± 2°C and 25°C ± 2°C under moist conditions for 60, 90 and 120 days with 10 seeds per replicate with 5 replicates in a completely randomized single factorial design. All combinations of stratification treatments were germinated in 100 x 15 mm petri dishes with several layers of sterilized blotting paper (Kimpak) which were kept moist by regular additions of sterile distilled water. Germination was monitored weekly by noting emergence of the radicle.

In experiment two, surface sterilized seeds were divided into 5 different treatments; control, cut, acid scarified for 4 hours and 6 hours, and mechanical scarification. Intact achenes were surface sterilized and placed in petri dishes as the control. The cut treatment was prepared by cutting the achenes in half transversely. The blunt end of the achenes containing the embryos were placed in petri dishes and germinated on moistened blotting paper as described above. Acid scarification treatments were conducted for four and six hours in glass beakers using Cross and Bevan's solution (1:2 $ZnCl_2$ • 2HCl w / v) (Skirvin *et al.*, 1990). Dry achenes were immersed in the acid solution for four or six hours in a volume ratio of approximately one part seed to three parts acid and occasionally stirred using a glass rod. Dry achenes were necessary as any trace of moisture allows the acid to penetrate the testa and damage the embryo. At the end of each treatment, the acid-seed mixture was poured over a mesh to drain the acid. Seeds were immediately immersed in a volume of cold water to prevent heating of the seed, and washed ten minutes in running water to ensure removal of acid residue before being placed in petri dishes.

Preliminary germination tests assessed potential acid damage to the embryos. Degradation of the seed coat and exposure of the embryo was microscopically observed at one hour intervals for 1 - 12 hours before choosing the four and six hour acid scarification treatments. The degree of degradation and acid penetration was detected by observing uneven thickness and blackening of the testa. The fifth treatment, mechanical scarification, was performed by abrading the seed coat with a medium grained sandpaper. The degree of abrasion was monitored by removing seed samples at different time intervals and visually assessing the thickness of the testa with the aid of a dissecting microscope. Mechanically scarified achenes used in the germination experiments had an area of their pericarp abraded to reveal the testa surrounding the embryo.

Seeds (10 / replicate with 5 replicates) were germinated in 100 x 15 mm petri dishes with several layers of sterilized blotting paper (Kimpak) initially moistened with a fungicide containing 2.5% oxine benzoate (No-Damp). The fungicide was prepared according to commercial recommendations. Following the first application of fungicide, deionized water was applied regularly to keep seeds moist throughout the experiment. All treatments were prepared under sterile conditions in a laminar flowhood and maintained in a controlled environmental chamber at 25°C ± 2°C with a 16 / 8 hour light / dark photoperiod. Seeds were monitored every second day for germination with the experiment being terminated after 11 weeks. Seeds were considered germinated once the radicle emerged. Germinated seeds were removed and planted in loam following the emergence of cotyledons. The seedlings were maintained in a misting chamber at 20°C ± 2°C with natural lighting. The chamber was intermittently misted every 30 min for 30 s. Experiments were of a replicated completely randomized single factorial design

In experiment two, cumulative germination of cut achenes was described using a modified Weibull function (Brown and Mayer, 1988b).

$$y = M [1 - e^{-(k (time - z))^c}]$$

The nonlinear model fitting procedure PROC NLIN in SAS 6.06 (SAS Institute Inc., 1984) was used to estimate parameters with boundaries obtained from the actual cumulative germination data. Initial values of M, k, c were estimated at 80.0, 0.01 and 4.0 respectively with boundary conditions as follows; $0.0001 < M < 100$, $0.0001 < k < 1$ and $0.0001 < c < 40$. Initial values and boundary conditions for z were not included in the Weibull function as actual values of zero during the lag phase prevented convergence of the model by creating a negative exponent. Zero germination values were replaced during the lag phase prior to initial germination when plotting the function.

In the third experiment, an *in vitro* technique was used in an attempt to germinate putative viable seeds. All seeds were surface sterilized following the protocol described above. Whole sterilized achenes were used as the control and placed on MS medium (Murashige and Skoog, 1962) solidified with Bacto-agar and supplemented with 3% (w / v) sucrose (appendix). Cut achenes were prepared under sterile conditions in a laminar flow hood by cutting the sterilized achenes transversely. The blunt end of the achene containing the embryo was placed in petri dishes also containing MS medium. Petri dishes were sealed with parafilm and maintained at ambient temperatures in the dark. Both treatments in this experiment were monitored daily for germination. The experiment was terminated after five weeks due to the degradation of the medium. Experiments were replicated three times in a completely randomized design.

2.3 Results

Separation of seeds by specific gravity proved to be a reliable test for ripe seeds, as 95% of seeds with high specific gravity were confirmed to be viable on visual examination,

while 90% of the seeds with low specific gravity were confirmed to be nonviable (Fig. 2.3.1). In contrast, viability tests on immature seeds revealed no clear separation of viable and aborted embryos. Upon visual examination, only 40% of immature seeds with high specific gravity possessed potential viability, while 40% of the seeds with low specific gravity contained aborted or nonviable embryos (Fig. 2.3.2).

In the initial experiment, 18 different dual stratification treatments of whole achenes at 5°C ± 2°C and 25°C ± 2°C in combinations of 60, 90, or 120 days failed to encourage substantial germination in either ripe or immature seeds. Although percentages were low, ripe seeds germinated when either the cold or warm stratification occurred first (Table 2.3.1 and 2.3.2). In contrast, immature seeds only germinated when exposed to long periods of warm stratification followed by cold stratification (Table 2.3.2). The majority of the replicates became contaminated after one week and were removed from treatment conditions before completion of the experiment to prevent the possibility of spreading the contamination.

In comparison with the other treatments in experiment two, the cut ripe seeds exhibited significantly higher germination rates over a shorter period of time (Fig. 2.3.3). The 6 hour acid treatment resulted in low germination rates following an extended lag period, while control, mechanically scarified and 4 hour acid treatments produced little germination of the achenes (Fig. 2.3.3). Short term cumulative germination data obtained from cut ripe seeds show a brief lag period followed by a rapid increase in germination and consequent leveling off to maximum germination (Fig. 2.3.4). The germination data from cut ripe seeds suggested the use of the Weibull function to represent cumulative germination. Maximum germination estimated by the asymptote (M) of the modified Weibull function was within approximately 2% of the actual total germination of 82% (Fig. 2.3.4). The lag parameter (c) was omitted from the Weibull function, as zero values

for achenes that had not yet germinated created a negative exponent that prevented the generation of a best fit equation by an iterative process, and therefore failed to meet the convergence criteria. Zero values for the lag phase were later included when plotting the curve predicted by the Weibull function (Fig. 2.3.4). The maximum rate of germination, or slope of the curve was estimated at 23 % per day. The shape parameter was estimated at 4.40, which is greater than the range of values that approximate a normal distribution by the Weibull function, therefore producing a negatively skewed distribution (Fig. 2.3.4). In general, the Weibull function correlated very closely with the actual data, with the fitted equation having a coefficient of determination (R^2) of 0.9995.

In the third experiment, *in vitro* techniques failed to encourage germination in both whole and cut achenes. All replicates became contaminated by fungi associated with the achenes, and therefore data are not available. Considering all experiments, the technique of cutting the achene in half and exposing the embryo was the only treatment to significantly enhance germination of wild rose. Seedlings produced from this treatment achieved 100% transplantation success when transferred to loam and maintained in a misting chamber. Within one month, seedlings could be removed from the intermittent misting conditions.

2.4 Discussion

Collection date of wild rose achenes greatly influences the ability to separate achenes into viable and nonviable categories. Achenes that were immature did not successfully separate into viable or nonviable categories in the preliminary viability tests (Fig. 2.3.2). Aborted embryos from immature achenes can maintain a relatively high percentage of their fresh weight as water (Bewley and Black, 1985), resulting in a misleading specific gravity. In contrast, ripe achenes clearly separated into viable and

nonviable categories in the preliminary viability tests (Fig. 2.3.1). As ripe achenes have already begun to dehydrate at the time of collection (Bryant, 1985) the aborted embryos are generally shrunken, creating a large air space within the achene. As a result, ripe achenes with aborted embryos have a low specific gravity and tend to float in water. It is therefore recommended that rose hips be collected once they are ripe as the viability is more predictable leading to the potential for separation of viable and nonviable achenes on a large scale.

Collection of ripe rose hips has also been recommended to avoid difficulties in germination (Hartmann and Kester, 1975). However, stratification experiments by King *et al.* (1983) showed that final germination rates (53%) for immature seeds of rose were higher than final germination rates of ripe seeds (35%). Unfortunately, literature on stratification techniques is often inconsistent. Densmore and Zasada (1977) reported that a long warm period followed by a short cold period is sufficient to remove dormancy and encourage germination in Alaska *Rosa acicularis*. In contrast, King *et al.* (1983) reported that use of longer periods of warm stratification greatly suppressed germination levels of both ripe and immature seeds of Alberta *Rosa acicularis*.

Another concept that has led to controversy is the importance of the warm stratification period in a dual stratification treatment. There is little evidence to indicate that high temperatures in themselves break dormancy, but Mayer and Poljakoff-Mayber (1982) demonstrated that high temperatures are responsible for a change in the seed coat structure of several species, thus causing a change in permeability. King *et al.* (1983) suggested that some species require a short period of warm temperatures (less than 60 days) to initiate the metabolic processes that are essential in germination. However, warm moist conditions can also be potentially detrimental to seed germination by enhancing bacterial and fungal activity (Pellet, 1973). The majority of the replicates in the dual

stratification experiments reported here, displayed fungal contamination which interfered with the germination of wild rose achenes. The extremely low germination rates presented in this study may be partially attributed to microbial damage to the embryo. As a result of contamination and low germination rates for both ripe and immature achenes, data from the dual stratification experiments (Table 2.3.1 and 2.3.2) are inconclusive. Therefore, the value of stratification as a technique to enhance germination does not appear to be justified for wild rose.

In the second experiment, control achenes possessed an intact seed coat which may have inhibited imbibition and possibly germination. Although physical resistance of the seed coat to the emergence of the radicle is unknown, it presents a potential restriction to germination. Although mechanical scarification treatments removed the pericarp and exposed the testa, imbibition may still have been prevented or slowed as germination did not occur. Villiers (1972) also reported no enhancement of germination of *Rosa* species following scarification treatment. However, Smithberg and Gill (1974) demonstrated that seed coat dormancy could be broken and germination time could be shortened by scarifying achenes of *Rosa* species. Acid treatments also degraded the seed coat, resulting in low rates of germination after an extended lag or imbibition period of 7 - 8 weeks (Fig. 2.3.3). Four hours of acid degradation may not have sufficiently weakened the seed coat to allow for germination. Six hour acid treatments digested more of the seed coat resulting in slightly higher germination (Fig. 2.3.3). Germination rates for this treatment were still not acceptable in terms of satisfying the primary objective of this experiment. Longer periods of acid digestion of the seed coat were tested and could possibly increase germination, but at the same time it was difficult to prevent acid damage to the embryo. The cut treatment increased germination four fold relative to the other treatments as it allowed complete exposure of the embryo encouraging rapid imbibition of water and subsequent germination. Studies by Ke *et al.* (1985) also indicated that nicking seeds of

Rubus to expose the embryo increased germination and decreased the time to maximum germination in comparison to seeds with an intact seed coat.

Embryo culture using *in vitro* techniques of both excised embryos and intact achenes did not facilitate germination as a result of 100% contamination. As blank plates were not contaminated, it appeared that contamination originated from a fungus within the pericarp despite surface sterilization. *In vitro* techniques have been successful in enhancing germination of strawberry (*Fragaria)* achenes without the problem of contamination (Miller *et al.*, 1992). Since the strawberry achenes that Miller and coworkers (1992) used were hybrids of selected cultivars maintained for breeding, the majority of microbial infections have been eliminated from the stock. In contrast, wild rose achenes were obtained from the field and thereby exposed to a variety of contaminants carried by the mother plant.

Although there is little data on dormancy of wild rose seeds in the literature, these experiments suggest that for ripe seeds in particular, the dormancy is possibly imposed by the seed coat. Seed coat dormancy is capable of inhibiting germination by depriving the embryo of oxygen and water as well as presenting a potential mechanical restriction to the embryo (Come and Thevenot, 1982). If the seed coat is removed and the embryo is exposed to appropriate conditions followed by germination, it is possible to assume that the dormancy is imposed by the seed coat. In contrast, if the embryo is still incapable of germinating it may be a result of embryo dormancy. As the technique of cutting ripe achenes in half and exposing the embryo greatly enhanced germination, it appears that the wild rose achenes exhibit seed coat dormancy at this stage of maturity. Tran and Cavanagh (1984) demonstrated that a majority of seeds become impermeable in the last stages of maturity and that the degree of impermeability is possibly related to seed moisture content. When shed, wild rose achenes possess a water content of approximately

15% of their fresh weight (Trau and Cavanagh, 1984). Although seed coats of immature seeds may be more permeable, putatively viable embryos are usually incapable of germinating as the developmental processes are incomplete. Apparently the immature embryo requires a period of warm moist conditions to enable development and subsequent germination (Bewley and Black, 1985). Based on the results of the stratification experiment, germination of immature achenes increased slightly when first exposed to warm temperatures followed by cold (Table 2.3.2), although the low germination rates obtained in these experiments do not support the use of stratification techniques to enhance germination of wild rose.

In the final stages of ripening in rose hybrid achenes, Mayer and Poljakoff-Mayber (1982) and Fordham (1990) detected an increase in the concentration of potential growth inhibitors such as cyanogenic glycosides and abscisic acid (ABA). These inhibitors are localized in tissues surrounding the embryo and are expected to contribute to dormancy by preventing development and growth of the embryo (Mayer and Poljakoff-Mayber, 1982). Past success of stratification in breaking dormancy is believed to be mediated by a shift in balance between growth inhibitors and growth promoters. Mayer and Poljakoff-Mayber (1982) detected a decline in ABA concentrations, liberation of hydrocyanic acid and synthesis of gibberellin and cytokinins during stratification. Apparently, the removal of the seed coat also results in similar changes in the balance between growth inhibitors and promoters (Ke *et al.*, 1985). By removing or cutting the seed coat, the inhibitors may be leached from the achene, leading to breaking of dormancy.

Data obtained from germination of cut ripe achenes suggested the potential use of the modified Weibull function to quantitatively describe cumulative germination. This nonlinear function permits accurate representation of cumulative germination over time and allows for comparison with other species as it is capable of reflecting maximum

germination, germination rate, lag in the onset of germination and the shape of the cumulative distribution. Other models are limited as they assume a normal distribution in the frequency of germination, whereas most germination tends to be skewed (Brown and Mayer, 1988a). They also cannot provide a satisfactory description of final germination as they lack the ability to asymptote (Brown and Mayer, 1988b). In contrast, the modified Weibull function is an exponential model that is capable of assuming an asymptote at final germination. Parameter M, allowed the function to fit a curve that was able to asymptote within 2% of the actual final germination of 82% (Fig. 2.3.4) rather than 100%. The Weibull function is also capable of estimating the rate of germination with the parameter k, which predicted a value of 23 % per day. The actual delay in approaching the asymptote increases as the parameter k decreases and therefore, the rate of germination will fall with k (Brown and Mayer, 1988b). The shape of the cumulative germination curve is determined by the shape parameter c in the Weibull function which allows the model to describe germination which has early or late germinating seeds. For this experiment, the shape parameter was estimated to be 4.40, producing a negatively skewed distribution which is consistent with the lag in actual data prior to germination (Fig. 2.3.4).

By understanding the parameters, the Weibull function was easy to use and flexible. The parameters have biological meaning that can be measured and quantitatively described. By providing boundary conditions and initial estimates for the parameters, convergence was achieved with an R^2 value of 0.9995. However, the absence of germination during the lag phase created difficulties by resulting in a negative exponent in the Weibull function. This situation prevented acceptable convergence of a best fit equation with the actual data. Therefore, the z parameter was eliminated from the function in order to obtain the Weibull distribution for cumulative germination beginning from the time of initial germination which corresponded to the third day after sowing. As

no germination occurred prior to this time, values for the Weibull distribution were assumed to be zero during the lag phase resulting in the expected sigmoid curve.

Effects of ripeness were significant in these studies as the results suggest that hip ripeness at the time of collection affects the relative germinability of wild rose. In the stratification experiments, ripe achenes yielded higher germination rates than immature achenes. As this conclusion contradicts previous studies, it is possible that climatic variability and the status of the mother plant may also be factors that affect germinability of wild plants. Apparently techniques to break dormancy using whole achenes such as stratification, scarification and acid treatments have little effect on wild rose seed dormancy and subsequent germination. It was found that in order to enhance germination, cutting achenes to directly expose the embryo gave the highest rates of germination. As this technique also enhanced germination in other rosaceous species with hard endocarps surrounding the embryo, it suggests that dormancy may be imposed by the seed coat. The cut achene technique described offers several advantages over traditional methods used to germinate rosaceous species. Inhibitors associated with the seed coat are readily diffused from the embryo, as well as allowing rapid imbibition of water and exchange of gases (Bewley and Black, 1985). Germination time is also shortened for the seed population allowing the evaluation of seedlings over a uniform age (Bewley and Black, 1985). Also, a higher percentage of seedlings are capable of surviving acclimatization and transplantation to the field when dormancy is broken by cutting the achene as evidenced by the 100% success in transplanting wild rose seedlings in a misting chamber.

Based on these conclusions, cutting achenes to achieve maximum germination can be a valuable method in providing a large number of seedlings for reclamation practices. The only major drawback appears to be that it is labor intensive. Therefore, despite the

success, other methods of propagation should also be assessed before adopting this technique.

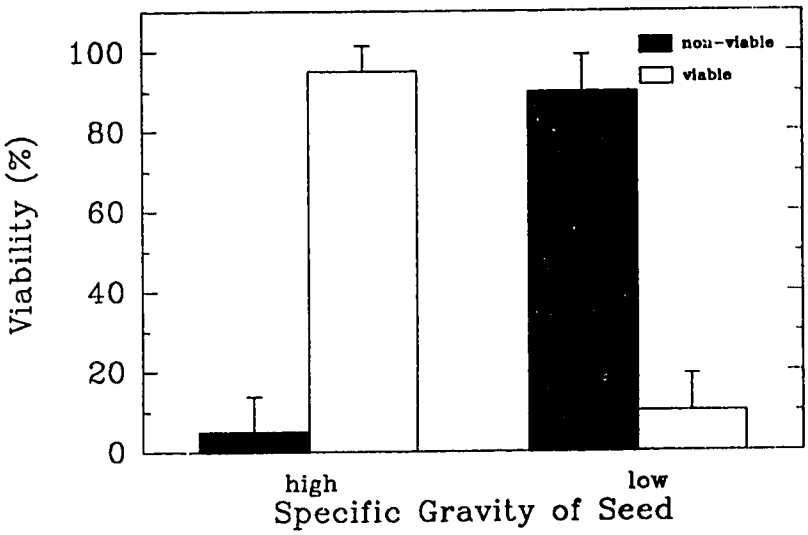

Fig. 2.3.1 Viability of ripe wild rose achenes separated by specific gravity and visually examined to determine the status of the embryo.

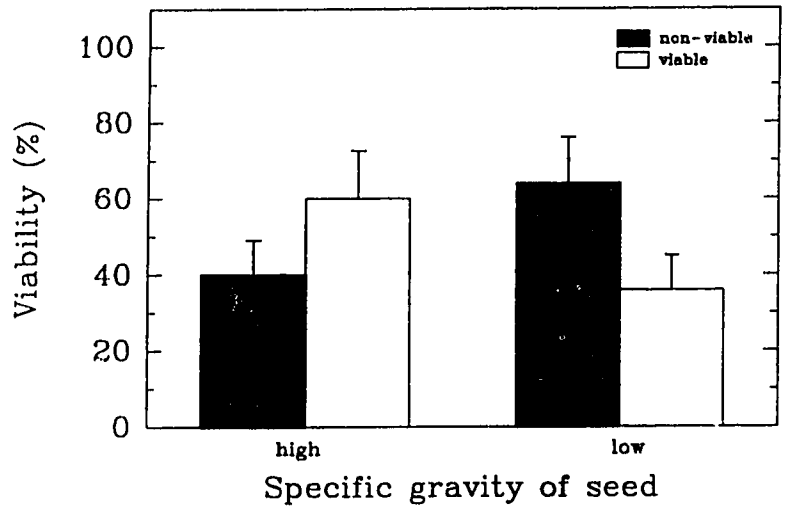

Fig. 2.3.2 Viability of immature wild rose achenes separated by specific gravity and visually examined to determine the status of the embryo.

Table 2.3.1. Percent germination (± s.e.) of ripe and immature achenes in response to dual stratification of 5°C ± 2°C followed by warm stratification at 25°C ± 2°C.

Dual stratification 5°C ±2°C / 25°C ±2°C (days)	Ripe Achenes (% germination)	Immature Achenes (% germination)
60 / 60	0 ± 0	0 ± 0
60 / 90	0 ± 0	0 ± 0
60 / 120	0 ± 0	0 ± 0
90 / 60	0 ± 0	0 ± 0
90 / 90	0 ± 0	0 ± 0
90 / 120	2 ± 2	0 ± 0
120 / 60	2 ± 2	0 ± 0
120 / 90	8 ± 3	0 ± 0
120 / 120	4 ± 2	0 ± 0

Table 2.3.2. Percent germination (± s.e.) of ripe and immature achenes in response to dual stratification of 25°C ±2°C followed by cold stratification at 5°C ± 2°C.

Dual stratification 25°C ±2°C / 5°C ±2°C (days)	Ripe Achenes (% germination)	Immature Achenes (% germination)
60 / 60	0 ± 0	0 ± 0
60 / 90	0 ± 0	0 ± 0
60 / 120	0 ± 0	0 ± 0
90 / 60	0 ± 0	0 ± 0
90 / 90	2 ± 2	0 ± 0
90 / 120	8 ± 3	0 ± 0
120 / 60	6 ± 2	6 ± 3
120 / 90	22 ± 13	0 ± 0
120 / 120	0 ± 0	10 ± 8

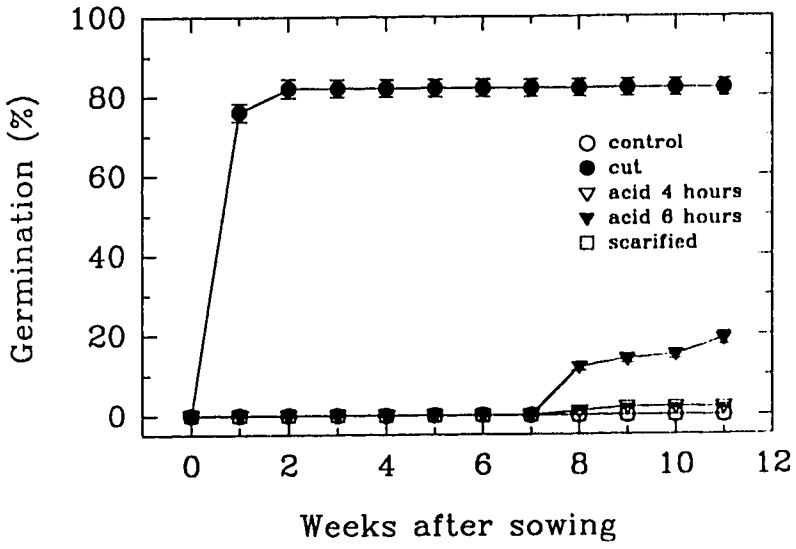

Fig. 2.3.3 Cumulative germination (± s.e.) for whole (control), cut, acid scarified (four and six hours) and mechanically scarified wildrose achenes.

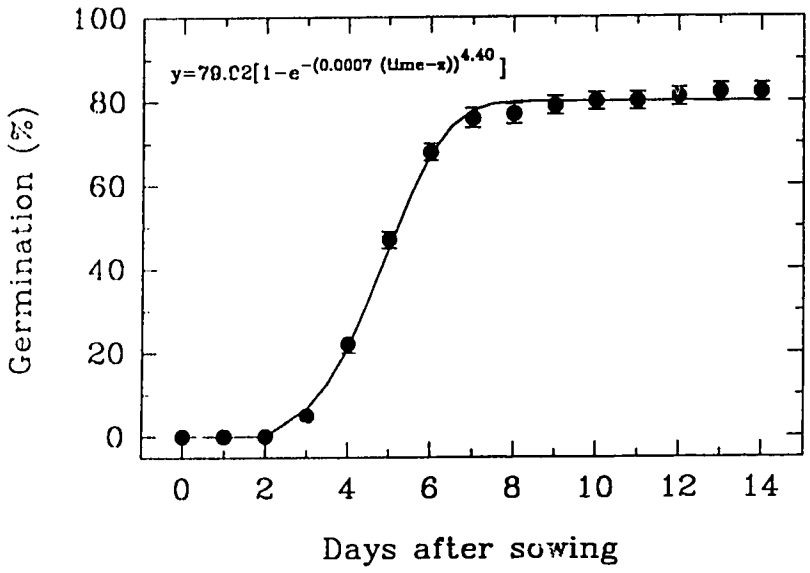

Fig. 2.3.4 Cumulative germination (± s.e.) of cut achenes over a 14 day experimental period. A modified Weibull function was fit to untransformed data (Brown, 1987) with a coefficient of determination (R^2) of 0.9995.

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3 Propagation of Wild Rose by Stem Cuttings

3.1 Introduction

For centuries plant propagators have sought ways to enhance rooting of woody cuttings. Traditional methods of grafting, budding and air layering are plagued by high labor costs and lengthy production time (Maynard and Bassuk, 1990), forcing the expansion of vegetative propagation studies in other areas. Although studies in non-vegetative propagation are encouraging, the method of using seed is subject to problems with genetic variability (Maynard and Bassuk, 1990) and dormancy, which result in poor or inconsistent seedling production. Micropropagation, which may eventually dominate the cloning market, also has problems with somaclonal variation, cost and technique prerequisites (Donnelly, 1990). Therefore, a simple, low cost, reliable method of enhancing the rooting of cuttings of woody species would still be of value.

Propagation of rose on a large scale by root cuttings has proven successful, although numerous disadvantages have contributed to this method's slow demise as a propagation method. Fedkenheuer (1987) found propagation of wild rose by root cuttings to be time consuming and labor intensive, resulting in elevated production costs. Obtaining root cuttings also presents a problem as collection of stock material disturbs the landscape and damages vegetation. As a result, propagating wild rose by stem cuttings would be more acceptable.

Success of rooting stem cuttings is based on a variety of factors including the physiological state of the stock plant, the propagation environment and the treatment applied to cuttings prior to propagation, such as the concentration of applied auxins (Newton *et al.*, 1992). The status of the stem at the time of cutting, whether it be

hardwood, softwood or semi-hardwood is thought to be a critical factor in rooting. Hardwood stem cuttings are considered dormant but still capable of rooting, while softwood stem cuttings are metabolically active (Hermesh and Cole, 1983). Semi-hardwood stem cuttings are generally considered to be in the later stages of activity leading into a dormant condition (Hermesh and Cole, 1983). Studies on the status of stem cuttings for both wild and cultivated roses are controversial with respect to which condition would be more suitable for rooting. Fedkenheuer (1987) found a higher percentage of rooting in hardwood wild rose stems, but still reported appreciable rooting in softwood stem cuttings. In contrast, Osbourne (1990) suggested that taking cuttings prior to flowering, while the stems are still classified as softwood, increases the percent rooting in wild rose. Osbourne (1990) also demonstrated that the position on the stock plant from which a stem cutting is taken is also relevant to root production. Shoots emanating from the base do not root as effectively, possibly because they maintain a proportionally lower level of carbohydrates compared to lateral shoots (Osbourne, 1990).

Other factors involved in the rooting of stem cuttings are the physio-chemical properties of the rooting media. An appropriate medium must provide adequate physical support, aeration, water holding capacity and drainage. The most common types of media include; sand, peat, loam and commercially prepared mixtures such as pearlite and metro mix. Size and shape of particles in the medium and the degree of compaction determine the amount and size of air spaces between particles (Peate, 1990). Air spaces in the medium are important as they permit penetration of roots and allow drainage of water. An appropriate medium should contain approximately 27% available air space throughout its volume to allow the developing root system to absorb oxygen and release carbon dioxide (Peate, 1990). A suitable media must also have a low salt content and a pH of 4 to 6 depending on the species being propagated (Peate, 1990). Despite these rather specific recommendations, Fedkenheuer (1987) were unable to identify a preferred

medium for enhancing rooting of wild rose stem cuttings. However, they did suggest that most particles should be coarse in texture, approximately 1 - 5 mm in size, with the inclusion of finer particles to maintain the water holding capacity.

In general, adventitious root formation occurs in three phases. The first phase involves de-differentiation of predetermined cells to act as mother cells for the root primordia (Hartmann *et al.*, 1989). De-differentiation is followed by initiation in which these cells divide and form the root primordium (Hartmann *et al.*, 1989). Finally, the primordium forms vascular connections and elongates, protruding through the surrounding tissue to form adventitious roots (Hartmann *et al.*, 1989). Physiological studies (Hemberg, 1954; Beck and Sink, 1974; Jarvis, 1986) have shown that auxin plays a central role in the developmental process of root initiation. During this stage, auxin must be supplied for roots to form. With cuttings that are easy to root, it has been assumed that endogenous auxin levels from buds or leaves are sufficient to promote adventitious root formation (Sagee *et al.*, 1992). On the other hand, woody cuttings that are difficult to root require an exogenous application of auxin to promote adventitious root formation (Sagee *et al.*, 1992).

The greatest advancement in the rooting of woody stem cuttings was the discovery of auxin (indoleacetic acid (IAA)), a naturally occurring root promoting substance (Thimann and Went, 1934). Later, indolebutyric acid (IBA) and naphthaleneacetic acid (NAA), two synthetic analogs of IAA, were also shown to have root inducing effects on cuttings (Maleike, 1990). Currently, IBA and NAA can be found as the active components of numerous commercially available rooting compounds. In contrast, IAA is infrequently used as a rooting compound as it readily breaks down in light (Read *et al.*, 1990) and can be readily metabolized by plants (Maleike, 1990). As IBA and NAA are synthetic auxins, they are foreign to the plants biosystem and therefore are not destroyed

as quickly (Maleike, 1990) which increases their potential to enhance the formation of adventitious roots.

In response to the demand for a simple, low cost method for enhancing the rooting of a woody species, the objective of this research was to develop efficient propagation methods for wild rose by identifying (1) propagating media, (2) hormone concentrations and (3) the best time to take cuttings that would be inducive to rapid rooting.

3.2 Materials and Methods

Lateral stem cuttings of wild rose were collected from Rainbow Valley in South west Edmonton six times throughout the year to obtain stems with different wood status. Cuttings were collected in mid October as a semi-hardwood sample, mid January and April as hardwood samples and June and August as softwood samples. Cuttings were taken from healthy field plants, which were visually inspected to detect any signs of disease such as leaf spots, rusts, mold or mildew, or physical damage caused by insects or herbivores. Field cuttings were brought into the greenhouse and trimmed to a length of 18 cm with a horizontal cut on the end to be inserted into the media. The base of the cuttings were dipped in 0, 0.1, 0.3 or 0.8% indolebutyric acid (IBA) rooting powder (Seradix - May and Baker Ltd.). Excess rooting powder was shaken off, and the cuttings were inserted 5 cm into root trainers filled with either sand (Canar Sand and Gravel), loam (Canar Sand and Gravel) or metro mix (W.R. Grace and Company of Canada Ltd.). Two sets of softwood cuttings were collected in August as a secondary experiment to assess the effect of defoliation compared to intact foliage on root production in stem cuttings. Foliage was entirely removed from one set of stem cuttings prior to planting, while the other set was planted with the foliage intact. The propagation procedure was performed as mentioned above.

Nine flats of root trainers were prepared by plugging the bottom with foam to prevent media from seeping out, while still allowing water drainage. Each flat, representing a single replicate had 12 rows of 5 root trainers per flat. Each flat was completely filled with one of the three media (sand, loam or metro mix) with three rows of cuttings for each hormone concentration (0, 0.1, 0.3 and 0.8% IBA) randomly arranged throughout the flat. Three replicates were prepared for each medium representing a 3 x 4 randomized factorial design. After the flats were filled with media they were lightly watered to facilitate planting of the cuttings. After planting, cuttings were immediately placed in a misting chamber with bottom heating at a temperature of 20°C ± 2°C. Distilled water was intermittently misted (30 s) on the cuttings every 30 min. Natural light was the only source of irradiation and therefore varied throughout the season. Cuttings were harvested after one month by washing the media from the roots to avoid damage. The number of roots were counted for each stem and removed to measure fresh root weights.

Although stem cuttings treatments were replicated they were not repeated over time. Furthermore, direct statistical comparison of results throughout the season is not valid because of differences in environmental conditions and physiological status of the cuttings which are confounded with season. Data collected for each season was separately analyzed using an ANOVA (Appendix 6.1). Therefore, the results presented can only be used to suggest possible trends in the propagation of wild rose through stem cuttings taken at different times of the year.

3.3 Results

Marked differences in rooting success were observed in stem cuttings of wild rose for each of the five harvest dates. Although statistical analysis is not possible, propagation

of softwood stem cuttings taken in June and August appeared to produce the highest root weights in all three media types, while cuttings for October and January produced the lowest root weights (Fig. 3.3.1 a - c). For the softwood cuttings taken in August and June, root weights increased with increasing concentrations of IBA applied to the base of stems (Fig. 3.3.1. a - c). In contrast, IBA had no effect on root production in hardwood stem cuttings taken in January, and limited root production in semi-hardwood cuttings taken in April (Fig. 3.3.1. a - c). Even an increase in rooting hormone did not enhance rooting of hardwood cuttings. Exogenous application of IBA in a powder form to the base of stem cuttings resulted in the formation of callus on all cuttings, from which roots eventually formed. The nature of the rooting medium did not consistently affect root weight of stem cuttings, although sand seemed to deter root growth in the softwood stem cuttings taken in August compared to the root growth in loam and metro mix (Fig. 3.3.1. a - c).

The overall effect of the medium on the number of roots produced in each of the five harvests is summarized in Table 3.3.1. Softwood stem cuttings appeared to produce a greater number of roots, irregardless of the media used, while hardwood stem cuttings produced the least number of roots (Table 3.3.1). At each harvest, the total number of roots produced in loam was not substantially different than the number of roots produced in metro mix. Although sand appeared to be less effective than loam and metro mix in encouraging root production, it still provided a suitable media for encouraging root growth.

The effect of defoliation was tested on wild rose softwood stem cuttings collected in August. Cuttings where the foliage was retained produced higher root weights than cuttings which had their foliage removed prior to experimentation (Fig. 3.3.2). This trend was consistent for all three types of media, with sand producing slightly lower root

weights than loam and metro mix. Increasing levels of exogenous IBA increased root weights in stem cuttings with their shoots intact, while IBA had little affect on root production in cuttings with foliage removed. Defoliated softwood cuttings had a response similar to that of hardwood cuttings by producing extremely low root weights under all treatment conditions.

Overall, the highest root production was found when stem cuttings were collected in a softwood state with foliage present at the time of propagation. A rooting hormone with the concentration of 0.8% IBA significantly enhanced root production in softwood cuttings, although the optimal level of IBA was not obtained. Therefore, the results suggest that the rooting of wild rose stem cuttings is influenced by the time of cutting and the concentration of exogenous rooting hormone, while rooting does not appear to be sensitive to the media types used in this experiment.

3.4 Discussion

With stem cuttings, the root system, which is part of the physical and physiological support for the plant has been removed. Therefore, the stem cutting must have the resources available and a metabolic state capable of regenerating a root system. As the physiological stage of the stock plant is a concern, stem cuttings were taken to represent hardwood, softwood and semi-hardwood conditions to detect any trends in the number of roots produced. The effects of rooting media and concentration of rooting hormones were also considered in root regeneration of wild rose stem cuttings.

These results suggest that softwood stem cuttings of wild rose are capable of producing the highest root weight in all three media used in this experiment. Softwood cuttings also responded to increasing IBA concentrations as evidenced by a corresponding

increase in root weight (Fig. 3.3.1). As softwood cuttings are taken from relatively new growth on the stock plant they tend to respond more readily to propagation treatments as they are in a metabolically active stage (Osbourne, 1990). Hybrid roses have been propagated by stem cuttings with optimal rooting occurring when cuttings were taken in early summer (Collicutt, 1992). In some cases, the ability to regenerate roots is absent in mature growth (Maleike, 1990). Although hardwood stem cuttings are often considered to be dormant and produce low root weights as reported here for wild rose, other researchers have achieved successful propagation using hardwood cuttings. Hermesh and Cole (1983) propagated wild roses by stem cuttings and recommended that dormant hardwood cuttings were the mostreliable source for propagation. Studies on species of *Cornus* and *Prunus* also supported the use of hardwood cuttings for propagation. Defoliation, weak root systems and stem rot were problems associated with root formation using softwood cuttings (Nirider, 1990). A possible explanation for the conflicting results reported in the literature is that the physiological status of the stem cutting is related to climatic conditions experienced by the stock plant (Collicutt, 1992). As the climatic conditions vary from one area to another as well as throughout the year, general conclusions become complicated with the use of field plants as stock material. Also, findings from one experiment with a particular species is not always applicable to another species as they have different structural and physiological characteristics.

In addition to the physiological status of the stem, the current literature also emphasizes the importance of rooting hormones in the production of roots on stem cuttings. In this experiment, an increase in root weight of softwood stem cuttings corresponded to an increase in the concentration of IBA, to a level of 0.8%. Higher concentrations of IBA should have been tested as the optimal level of rooting hormone was not detected in the range used in this experiment. Effective concentrations of IBA for promoting root formation of easy to root plants such as *Phaseolus spp.* have been

reported at 0.6 - 0.12% (Hemberg, 1954), while difficult to root woody plants such as *Tilia spp.* and *Prunus spp.* require 0.10 - 0.40% IBA to promote rooting (Chong and Daigneault, 1990). Therefore, it appears that the rooting response in stem cuttings to IBA peaks at different concentrations depending on the species involved. Chong and Daigneault (1990) reported that IBA concentrations greater than 0.40% were detrimental to the propagation of cuttings due to IBA phytotoxicity. For woody species, exogenous applications of 0.20 - 0.40% IBA results in basal injury of the stem cutting (Lane, 1990). The additional application of auxin induces mitoses and cell elongation resulting in the formation of callus at the site of basal injury (Moore, 1990). Cuttings generally accumulate soluble carbohydrates at the base of the stem prior to the formation of root primordia (Jarvis, 1986). Therefore, it has been suggested that carbohydrates are the principal source of energy during rooting as they are present in greater concentrations than alternative energy sources (Haissig, 1986).

Endogenous auxin is transported basipetally, with the rate of transport declining with age of the plant (Jarvis, 1986). This may explain the different responses of softwood and hardwood cuttings. If softwood cuttings are taken from new growth with an active auxin transport system, it would be expected that root formation would be vigorous. Also, with the application of exogenous auxin in addition to endogenous auxin, there would be a higher overall auxin concentration to promote root initiation. In contrast, if the hardwood cuttings have an inadequate auxin transport system, the majority of the auxin available would come from the exogenously applied auxin. Therefore, it is possible that shoots containing the greatest amount of auxin originally, will produce the largest number of roots because the combined concentration of endogenous and exogenous auxin is sufficient to promote root initiation (Beck and Sink, 1974). The literature also suggests that high concentrations of auxin which are required to initiate root formation are inhibitory to later stages of root development, implicating auxin metabolism during the

course of primordia formation (Jarvis, 1986). In this experiment the highest concentration of supplied IBA was 0.8%. This concentration did not appear to inhibit root formation as root weights were still increasing at this hormone level (Fig. 3.3.1). Therefore, higher concentrations of supplied IBA should be studied to determine the maximum level for root formation in the propagation of wild rose.

Defoliation of softwood stem cuttings prior to experimentation prevented substantial rooting despite the addition of exogenous IBA (Fig. 3.3.2). Removal of the buds and leaves may have eliminated the endogenous auxin supply, leaving only the supplied auxin concentration to promote root formation. For difficult to root species, such as wild rose, the IBA concentrations used in this experiment may not have been sufficient to enhance rooting of leafless cuttings. In contrast, increasing levels of exogenous IBA concentrations promoted the rooting of leafy softwood stem cuttings (Fig. 3.3.2. a - c). Similar trends in root production occurred in defoliated softwood cuttings and hardwood cuttings. As both were leafless prior to experimentation, a possible explanation can be suggested by assessing the importance of the presence of foliage during propagation. Previous studies have demonstrated that transport of sucrose from leaves to the rooting region is enhanced during early stages of initiation (Jarvis, 1986). Exogenous auxin is believed to enhance sugar transport by influencing sugar content in the leaves (Jarvis, 1986). Therefore, if leaves are not present at the time of cutting preparation, sugar transport is lower despite the application of supplied auxin. Low sugar levels at the site of root initiation decreases the potential for root regeneration (Jarvis, 1986).

The role of the leaf may also be essential in controlling the water balance of the cutting (Newton *et al.*, 1992). It has been suggested that an optimum leaf area exists for rooting, where the balance between water loss and photosynthesis is optimized (Newton *et al.*, 1992). Therefore, environmental conditions that reduce water loss must be

considered when propagating stem cuttings. Intermittent misting of cuttings was applied in this experiment to prevent elevated transpiration and respiration rates that would deplete the carbohydrate reserves which are necessary to encourage rooting (Maleike, 1990). Misting of the cuttings kept humidity levels close to 100 % and created a constant water film on leaves. Water absorbed through the leaves decreases evaporative demands from the leaves and reduces water loss thereby conserving internal water (Maleike, 1990). However, it is important to maintain appropriate misting levels as excess water can leach nutrients from the leaves as well as increase the incidence of rotting and disease (Osbourne, 1990). Poor rooting in studies on *Phytolacca* have been associated with wilting and desiccation of cuttings as a result of high temperature and low relative humidity (Demeke *et al.*, 1992). Also, if cuttings are subjected to bottom heating as in this experiment, enough water must be applied to prevent rooting media from drying out. Several studies have assessed the value of bottom heating in enhancing root formation. Current literature suggests that bottom heating encourages callus formation at the base of stem cuttings that have been treated with rooting hormones (Moore, 1990). Quick callusing properties are expected to lead to rapid root initiation of stem cuttings (Moore, 1990).

Rooting medium is also an important factor involved in successful propagation of stem cuttings. The rooting media should satisfy certain physical, chemical and biological criteria as well as those of availability, consistency of quality, low cost and ease of use (Peate, 1990). Three common rooting media used in this experiment were sand, loam and metro mix. Although there did not appear to be a major preference with respect to rooting media, the general trend reported here was that sand was slightly less effective than either loam or metro mix at promoting root formation for all harvests, except for the semi-hardwood cuttings collected in October (Table 3.3.1). Perhaps the slightly lower root weights in sand can be attributed to the lack of cation exchange capacity and low

water holding capacity (Peate, 1990). As mentioned previously, media used with misting must be capable of drainage, while media on heating beds must be kept wetter (Peate, 1990). Therefore, media with an adequate mixture of coarse (1 - 4 mm) and fine (< 1 mm) particles such as loam and metro mix, would provide an appropriate rooting environment for wild rose stem cuttings propagated using intermittent mist and bottom heating.

Unfortunately, currently available information on propagation of wild rose stem cuttings is contradictory making general conclusions unreliable. Results produced under laboratory conditions may be reproducible on a small scale but not necessarily feasible for large scale commercial production. In addition, adventitious root formation is regulated by complex interactions between endogenous and exogenous factors which affect the developmental stages of root formation. The data presented here demonstrate how the environment which supports and facilitates rooting of cuttings has a profound influence on the success or failure of root regeneration. The trends reported here for wild rose show that it is important to take cuttings from new growth, use high concentrations of supplied IBA (0.8%) and retain foliage on the cuttings to encourage root formation. Despite the present success of propagating wild rose by softwood stem cuttings, the technique may be unpredictable as it is dependent on climate and availability of healthy stock material. Therefore, it is still important to consider alternative methods for propagation which are not dependent on field material.

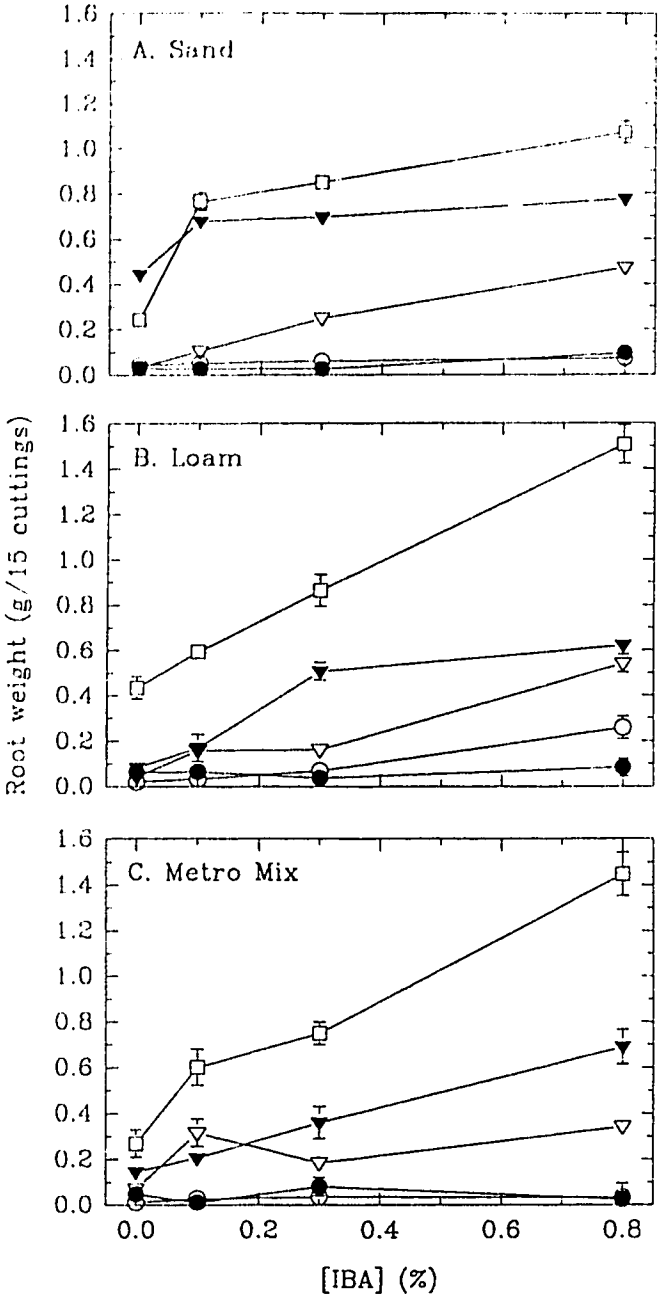

Fig. 3.3.1 The influence of rooting media and hormone concentrations (IBA) on root weight of stem cuttings of different physiological status. Values presented are the mean root weights of three replicates (15 cuttings) (± s.e.). □ August (softwood), ▼ June (softwood), ∇ April (hardwood), ● January (hardwood) and ○ October (semi-hardwood).

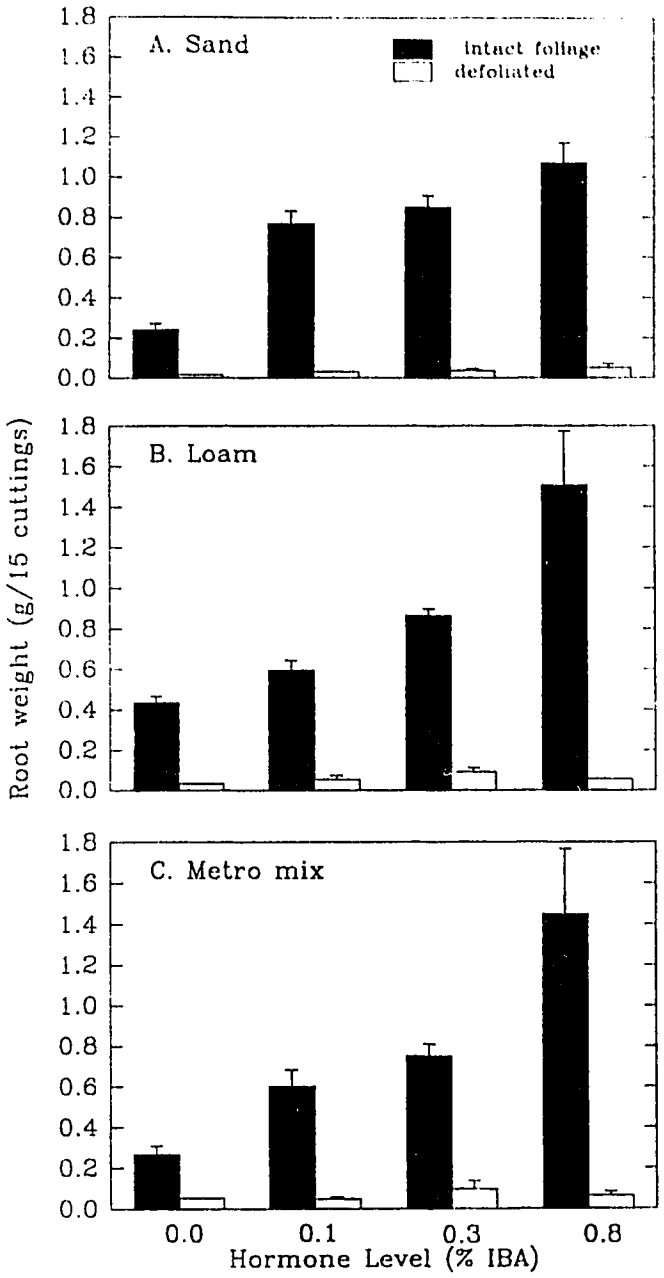

Fig. 3.3.2 The effect of defoliation of softwood (August) stem cuttings on root weights. Stem cuttings were treated with rooting hormone (IBA) and propagated in three rooting media; sand, loam and metro mix. Values presented are the mean root weights (± s.e.) of three replicates (15 cuttings).

Table 3.3.1 The influence of cutting time and rooting media on the total number of roots produced in wild rose stem cuttings. Values presented are the total number of roots produced for three replicates (45 cuttings) for each harvest date in a specific medium.

Time of Cutting and Wood Status	Sand	Loam	Metro mix	Total # of roots / cutting time
January (hardwood)	169	210	239	618
April (hardwood)	512	568	611	1691
June (softwood)	713	779	804	2296
August (softwood) (intact foliage)	886	911	920	2717
August (softwood) (defoliated)	317	396	406	1119
October (semi-hardwood)	275	362	174	812
Total # of roots / medium	2872	3227	3154	

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4 Micropropagation of Wild Rose Through Tissue Culture Techniques

4.1 Introduction

Traditionally, roses are propagated by budding, grafting or by rooted cuttings. More recently however, tissue culture of the genus *Rosa* has become an alternative method of propagation. There are numerous studies involving the *in vitro* culture of commercial roses using axillary buds (Davies, 1980; Bressan *et al.*, 1982) shoot tips (Jacobs *et al.*, 1970; Hasegawa, 1980), embryos (Burger *et al.*, 1990) and anthers (Tabaeezadeh and Khosh-Khui, 1981). However, to date there has only been one study on the *in vitro* propagation of wild rose species. Micropropagation comparisons were made between *Rosa hybrida* L. and two wild rose species, *Rosa canina* L. and *Rosa damascena* L. by Khosh-Khui and Sink (1982). They reported that although hybrids produced callus more readily and rooted more easily from *in vitro* proliferated shoots, than the wild rose species, it was still feasible to propagate wild rose *in vitro* once these difficulties were alleviated by the development of a proper protocol for wild rose (Khosh-Khui and Sink, 1982).

Different cultivars of roses seem to have different requirements for media and growth regulators in order for callus proliferation and organ formation to occur (Arnold *et al.*, 1992). For example, Noriega and Sondahl (1991) reported that a medium developed for *Rosa hybrida* L. produced different forms of callus from a variety of explants. Arnold *et al.* (1992) found no consistent response among multiplication rates and growth regulator requirements for different cultivars. Although these results indicate that not all species of roses proliferate equally well with the same medium or growth regulators, it is generally agreed that cytokinins and auxins are components of the medium which have the greatest affect on the performance of the explant (Dirr and Haeussler, Jr., 1987).

Composition of the culture medium is an important factor in successful establishment of a callus culture. The most commonly used medium for micropropagation is a basal medium developed by Murashige and Skoog in 1962. Modifications to this standard medium allow it to be used with a wide variety of plants. The Murashige and Skoog (MS) media provides components essential for growth, such as sucrose which is the most commonly used carbon energy source for *in vitro* culture. Sucrose also plays an important role in maintaining suitable osmolarity of the culture medium (Hu and Wang, 1986). Organic components also have a catalytic function and are supplied in the form of vitamins such as inositol, nicotinic acid, pyridoxine and thiamine and amino acids such as glycine or cysteine (Murashige and Skoog, 1962). Inorganic components include nitrogen, phosphorus, sulphur, potassium, calcium, sodium, magnesium and chlorine and micro-nutrients such as iodine, boron, molybdenum, cobalt, manganese, copper, zinc and iron (Murashige and Skoog, 1962). In addition to a catalytic function, the primary physiological role of the inorganic components includes maintenance of the membrane potential, osmotic balance and cofactor roles in enzymatic reactions (Jackoby and Pastan, 1979).

The current literature also suggests that there is a lower limit to the size of callus capable of growth after isolation from the parent callus (Reinert and Bajaj, 1977). Few data are available from callus grown on semisolid media, but it has been suggested that cell division does not occur in callus suspension cultures of *Nicotiana spp.* below critical concentrations of 60,000 cells per ml (Jakoby and Pastan, 1979). Formation of callus is often a preliminary step required for regeneration of plantlets from a somatic explant. Callus can be defined as an amorphous mass of actively dividing thin-walled parenchyma cells arising from proliferating cells of parent tissue (Dodds and Roberts, 1982). Under the influence of the appropriate ratio of cytokinin and auxin, callus has the potential for organogenesis which is the ability to produce organs such as roots and shoots *in vitro*

(Dodds and Roberts, 1982). By lowering the ratio of cytokinin relative to auxin, roots can be induced to form on the callus (Burger *et al.*, 1990). Reversing this ratio encourages the formation of shoots (Burger *et al.*, 1990). Therefore, depending on the ratio of growth regulators, organogenesis in callus is suspected to occur by initiation of the organ primordium (Reinert and Bajaj, 1977). Plants regenerated from tissue that has passed through a callus stage have the highest probability of expressing somaclonal variation (Schneider *et al.*, 1992). This is a result of the callus cultures undergoing numerous cell divisions during which natural mutations occur (Schneider *et al.*, 1992).

Depending on the goal of the research, *in vitro* propagation of plantlets can be achieved either directly from totipotent cells or via the intermediate step of callus production (Thomas and Davey, 1975). Totipotent cells are thought to contain all the information necessary to differentiate into embryo-like structures which can be influenced to follow the developmental sequences that occur in whole plants (Thomas and Davey, 1975). Although no ideal culture system yet exists, other studies have found it possible to break a complex multicellular organism into individual components and regenerate healthy plantlets from these cells (Schneider *et al.*, 1992). Therefore the overall objective of this particular research was to determine an appropriate culture system for *in vitro* propagation of wild rose. Determining the correct ratio of growth regulators to induce and maintain callus on leaf explants is the first step to organogenesis and plantlet production.

4.2 Methods

Rose hips were collected in September from field plants of wild rose. Achenes were removed from hips and surface sterilized in a laminar flow hood. Sterilization involved a 30 s treatment with 50% ethanol, followed by a rinse in sterile deionized water

with a few drops of Tween as a wetting agent. Achenes were then placed in 100 ml of 0.5% (w / v) sodium hypochlorite for 10 min with occasional agitation. Finally, the seeds were rinsed three times with sterile deionized water. Attempts to expose the embryo by removing the seed coat failed as the seed coat was too hard and securely attached. Instead, achenes were cut in half transversely or left intact for the control. Both were placed on modified semisolid MS culture media (Appendix 6.2) and left in the dark at 25°C ± 2°C.

In experiment two, wild rose leaves were collected from vigorous field plants and visually inspected for disease. Healthy leaves were surface sterilized in a laminar flow hood for 15 min with occasional agitation in 100 ml of 0.5% (w / v) sodium hypochlorite with a few drops of Tween added as a wetting agent. Finally, the leaves were rinsed three times with sterile deionized water. Leaf discs, which included the midvein, were aseptically excised with a sterile 6 mm diameter cork borer. Sterile leaf discs were placed with the adaxial surface in contact with a modified semisolid MS culture medium (Appendix 6.2) and left in the dark at 25°C ± 2°C.

For callus initiation and establishment, intact embryos, cut embryo cultures and leaf explants were aseptically cultured on a modified MS medium (Appendix 6.2) supplemented with 6 - benzylaminopurine (BAP) at 0, 0.25, 1.0, 2.0, 3.0 and 4.0 mgL^{-1} and alpha - naphthaleneacetic acid (NAA) at 0, 0.05, 0.10, 0.20, 0.40, 0.60, 0.80 and 1.0 mgL^{-1} in a dose response series. The pH of all media was adjusted to 5.8 and solidified with 8 gL^{-1} Bacto-agar (BDH, Ltd.). Each growth regulator in factorial combination was repeated twice using four embryos or explants per plate and four plates per replicate. All culture plates were sealed with parafilm to prevent external contamination. Callus initiation and development was monitored four times over an eight week incubation period. A one mm^2 grid was used to provide a measurement of establishment by counting

the total number of squares per plate that the callus covered. Each experiment was repeated twice and Duncan's multiple range test was used to make pairwise comparisons in order to separate the treatments (Appendix 6.3).

Experiment three was performed to increase the growth rate of callus once successful establishment on leaf explants was achieved. Healthy similar size pieces of callus were aseptically transferred from culture dishes to 150 ml Erlenmeyer flasks containing 50 ml semisolid modified MS media (Appendix 6.2) supplemented with BAP at 2.0, 3.0, 4.0, 6.0 and 9.0 mgL^{-1} and NAA at 0.40, 0.60, 0.80 and 1.0 mgL^{-1} in a dose response series. The pH of the media was adjusted to 5.8. Each factorial combination of growth regulators was repeated twice using four callus pieces per flask and four flasks per replicate. Initial size estimates of the first transfer callus pieces were taken by measuring the height and percent cover of the callus. Every four weeks for twelve weeks a growth estimate of the callus was taken followed by subculture of callus pieces to fresh media with the same growth regulator concentrations. This transfer process was repeated three times with the entire experiment being repeated twice. The data collected from the three replicates was analyzed using a Duncan's multiple range test to make pairwise comparisons in order to separate the treatments (Appendix 6.4).

4.3 Results

Attempts to establish callus in experiment one, from both intact and cut achenes were unsuccessful. One hundred percent contamination resulted, despite the sterilization procedure used in this experiment. In experiment two, callus establishment on leaf discs was initially observed along the cut edges of the leaf and along the midvein. In both experiments callus appeared as creamy-yellow, irregular masses of cells. Although, some inocula were soft and friable, others were compact and hard.

In experiment two, induction of callus on leaf discs occurred in petri dishes over a range of growth regulator concentrations. Analysis of variance (ANOVA) indicated that auxin (NAA) concentrations had a greater affect in influencing callus induction and growth than cytokinin (BAP) concentration. Callus measurements two weeks after innoculation revealed higher growth at NAA concentrations of 0.8 and 1.0 mgL^{-1} (Fig. 4.3.1 a). The Duncan's multiple range test indicated that the mean growth at the higher NAA concentrations were significantly different from the mean growth at lower NAA concentrations (Appendix 6.3). During the second time interval (2 - 4 weeks after innoculation), callus exhibited inconsistent growth rates (Fig 4.3.1 b) and all treatments appeared to have a similar capacity to generate callus. During the third time interval (4 - 6 weeks after innoculation), callus growth continued to respond to the higher levels of NAA with 0.8 mgL^{-1} producing the highest mean growth and the two lowest NAA concentrations of 0.1 and 0.2 mgL^{-1} producing the smallest mean growth (Fig 4.3.1 c). The fourth and final time interval (6 - 8 weeks after innoculation), revealed the most dramatic increase in callus growth with increasing NAA concentrations (Fig 4.3.1 d).

Concentrations of BAP did not appear to have a consistent influence on establishment and growth of callus in petri dishes, other than the lowest BAP concentration of 0.25 mgL^{-1}. This concentration of cytokinin was not effective in promoting any substantial growth in callus when compared to the higher concentrations of BAP (Fig 4.3.1 a - d). From these data it appears that higher auxin levels of 0.8 to 1.0 mgL^{-1} and cytokinin levels greater than 0.25 mgL^{-1} are required to initiate callus on wild rose leaf discs in petri dishes, although an optimal combination of growth hormones was not identified. The data also reveals a continual increase in callus growth over time (Fig 4.3.1 a - d).

In experiment three, attempts were made to enhance the growth of callus in Erlenmeyer flasks through three subcultures over a range of growth regulator concentrations. Following the initial subculture, there was no apparent influence of the auxin : cytokinin ratio (Fig 4.3.2 a). All combinations of growth regulators appeared to have a similar capacity to promote callus growth. Duncan's multiple range test supported this conclusion, as it was unable to separate the influence of the different concentrations of growth regulators at the time of the first subculture (Appendix 6.4). Following the second subculture, a distinct trend began to appear as evidenced by a decrease in callus growth with higher NAA concentrations (Fig 4.3.2 b). Callus growth curves from the third subculture revealed a similar trend, with a drop in callus growth as NAA levels increased (Fig 4.3.2 c). Overall, highest callus growth occurred at 4.0 mgL^{-1} NAA with no significant separation of BAP levels other than the highest concentration of BAP (9.0 mgL^{-1}) which consistently inhibited growth of callus. Omitting either or both hormones resulted in the absence of callus establishment on the leaf discs of wild rose.

4.4 Discussion

As conventional propagation techniques are becoming less suitable for mass production of woody plants, an alternative method for propagating species such as wild rose may involve the use of tissue culture regeneration. Currently, *in vitro* propagation techniques have been found to be capable of regenerating plantlets from callus for several species of *Rosaceae* including *Rubus* (McNicol and Graham, 1990; Cousineau and Donnelly, 1991), *Prunus* (James *et al.*, 1984), *Malus* (Korban *et al.*, 1992) and *Rosa* (Noriega and Sondahl, 1991; Arnold *et al.*, 1992; Rogers and Smith, 1992). In this study, successful induction of cell division resulted in the appearance callus around the cut edges of leaf discs following inoculation onto an agar medium. Callus formation occurred at the periphery of the leaf explants in response to injury during excision and the influence of

exogenous growth hormones in the medium. The dividing tissue is often referred to as a wound cambium in which successive divisions result in the cells becoming small and cytoplasmically dense, a phenomenon known as de-differentiation (Thomas and Davey, 1975). As new cells are formed by mitosis and cytokinesis, the callus mass continues to increase in size and eventually covers the entire explant (Thomas and Davey, 1975). Callus growth rates, appearance and texture are related to several factors including the composition of the culture medium.

As the regeneration of plants from isolated cells and tissues is under hormonal control, the balance of exogenous hormones required to induce and maintain callus growth depends on the balance of endogenous growth regulators present in the tissue at the time of excision from the plant (McNicol and Graham, 1990). Growth requirements frequently change during culture, requiring alterations in the concentrations of growth regulators in the medium. Auxins and cytokinins are crucial for stimulation of cell division and differentiation of tissues cultivated *in vitro* (Kaminek, 1992). Results reported here indicate the importance of growth regulators such as NAA and BAP during callus induction and growth. Initial callus induction appeared to require higher concentrations of exogenous NAA and BAP than growth and maintenance of callus did (Fig. 4.3.1 a - d and Fig. 4.3.2 a - c). Results given in Fig. 4.3.2 b and c show that increasing concentrations of NAA caused a significant inhibition of callus growth. These observations are in agreement with those previously reported by Pilet (1971) who found that higher auxin concentrations inhibited the growth of callus in *Rubus* tissue culture. A possible explanation for inhibiting callus growth with increased levels of NAA, is that the cells may have become habituated. Cells of well established callus tissues sometimes undergo spontaneous change after continuous subculture which is reflected by alteration in their requirements for exogenous hormones (Thomas and Davey, 1975). The literature suggests that habituated cells are able to synthesize relatively large amounts of auxins

which could account for their independence of exogenously supplied hormones (Thomas and Davey, 1975). The method by which habituated cells synthesize auxins is unknown, although increasing concentrations have been detected in *Rubus* tissue that was maintained over numerous subcultures (Thomas and Davey, 1975).

Some researchers have suggested that cytokinin is more important than auxin in regulating performance of the explant. In some cases, auxin has been omitted from the induction media (Dirr and Haeussler, Jr., 1987). In contrast, this study suggests that auxin concentrations were influential in enhancing callus induction and growth. Duncan's multiple range test was able to separate the mean growth of callus in response to auxin concentrations, while the effect of cytokinin on the growth of callus appeared to be inconsistent. Perhaps it is more appropriate to suggest that as auxins stimulate cell expansion and cytokinins stimulate cell division, a combination of the two growth regulators is required (Kaminek, 1992). Omitting either NAA or BAP resulted in no callus formation on wild rose leaf discs. The optimum concentration range of NAA and BAP for callus induction on wild rose leaf explants was 0.6 to 1.0 mgL^{-1} and 1.0 to 4.0 mgL^{-1} respectively. The optimum concentration range of NAA and BAP for callus growth and maintenance of subcultures was 0.4 mgL^{-1} and between 2.0 and 4.0 mgL^{-1} respectively. Although this study did not succeed in regenerating organs from the callus, further work in this area could be continued now that a range of growth regulators has been established for callus production. By varying the hormone ratio, organogenesis from callus should be possible. A high auxin : cytokinin concentration ratio in the culture medium induces rooting, while the reverse ratio favors bud and shoot formation (Skoog and Miller, 1957).

Despite successful induction of callus, a decline in growth may follow sub culturing as the size of the inocula is critical in maintaining continued cell divisions.

Dodds and Roberts (1982) suggest that callus needs to be 5 - 10 mm in diameter and weigh approximately 20 - 100 mg in order to subculture. Inocula smaller than this requires a longer period of time to establish substantial callus growth (Dodds and Roberts, 1982) and also tends to have less regenerative ability (Reinert and Bajaj, 1977). On the other hand, if callus inocula are too large, the incidence of microbial contamination increases. As bacterial contamination is one of the major problems of plant tissue culture, rigorous sterilization techniques are employed. Although, Leifert and Waites (1992) suggest that the majority of bacteria are introduced during aseptic handling of the plant material. They have demonstrated that different plant species grown *in vitro* create distinct environments inside the culture vessels which in turn allow or prevent the growth of different bacteria (Leifert and Waites, 1992). Medium pH and release of different compounds by the explant are suspected factors which might influence bacterial growth (Leifert and Waites, 1992).

Another factor which requires consideration with respect to callus induction and growth are the different environmental conditions the callus is subjected to. Callus growth was slower during initial induction and growth in petri dishes, while callus growth was more rapid during maintenance of growth in 150 ml Erlenmeyer flasks. A possible explanation for the slower growth in the petri dishes in addition to the affect of the growth regulators, is that there is less oxygen and nutrients from the medium available to support the cells.

An optimum culture time can be determined by assessing the physical appearance of the callus. Brown or necrotic callus is the sign of localized cell death which can potentially spread throughout the entire cell mass (Skirvin *et al.*, 1990). Discoloration or browning of the cells is a result of the oxidation of polyphenolic compounds (Skirvin *et al.*, 1990). This condition corresponds with a decline in callus growth as a result of

nutrient depletion of the medium and oxygen starvation of the central cells of the tissue (Skirvin *et al.*, 1990). Repeated sub culturing in the dark was used to eliminate this problem, while at the same time increasing the amount of cellular material produced from a single explant. Culturing in the dark also prevents numerous photochemical modifications to the culture media, such as the degradation of media components (Hu and Wang, 1986), therefore maintaining an environment that is able to support tissue growth.

From this study and previous studies, it can be concluded that medium composition, including growth regulators, environmental conditions and plant species all contribute to the success or failure of micropropagation. Therefore a holistic view of cellular growth requirements appears most appropriate, where all variables that affect growth are viewed a single complex interacting set (Jackoby and Pastan, 1979). Based on the findings of this study, it is possible to make a general recommendation for the range of auxin and cytokinin that should be used in the initiation and growth of callus from wild rose leaf explants. The *in vitro* process may offer an alternative to conventional vegetative propagation of wild rose, but further research is required for revegetation.

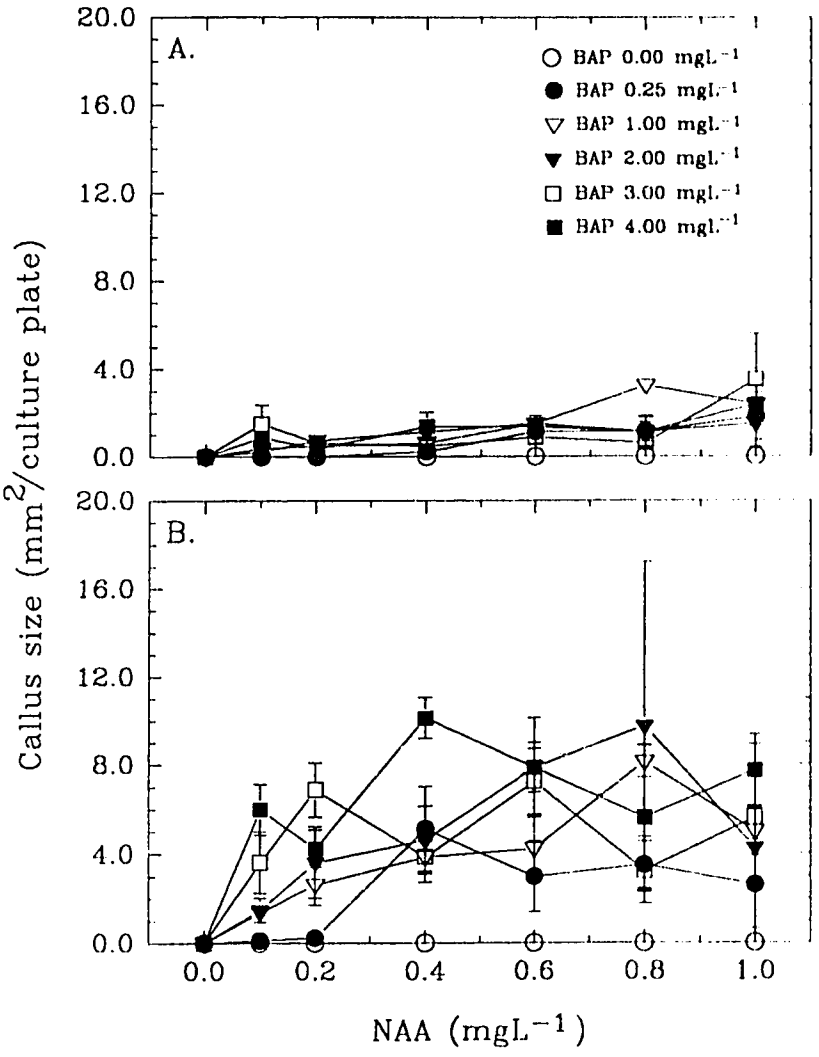

Fig. 4.3.1 The influence of a dose response series of the auxin, naphthaleneacetic acid (NAA), and the cytokinin, benylaminopurine (BAP), on callus induction and growth over an eight week incubation period. Hormones were supplied in a semisolid medium. Values presented are the mean callus size (± s.e.) of four replicates. (A) Callus induction two weeks following initial inoculation, (B) Callus size four weeks after inoculation.

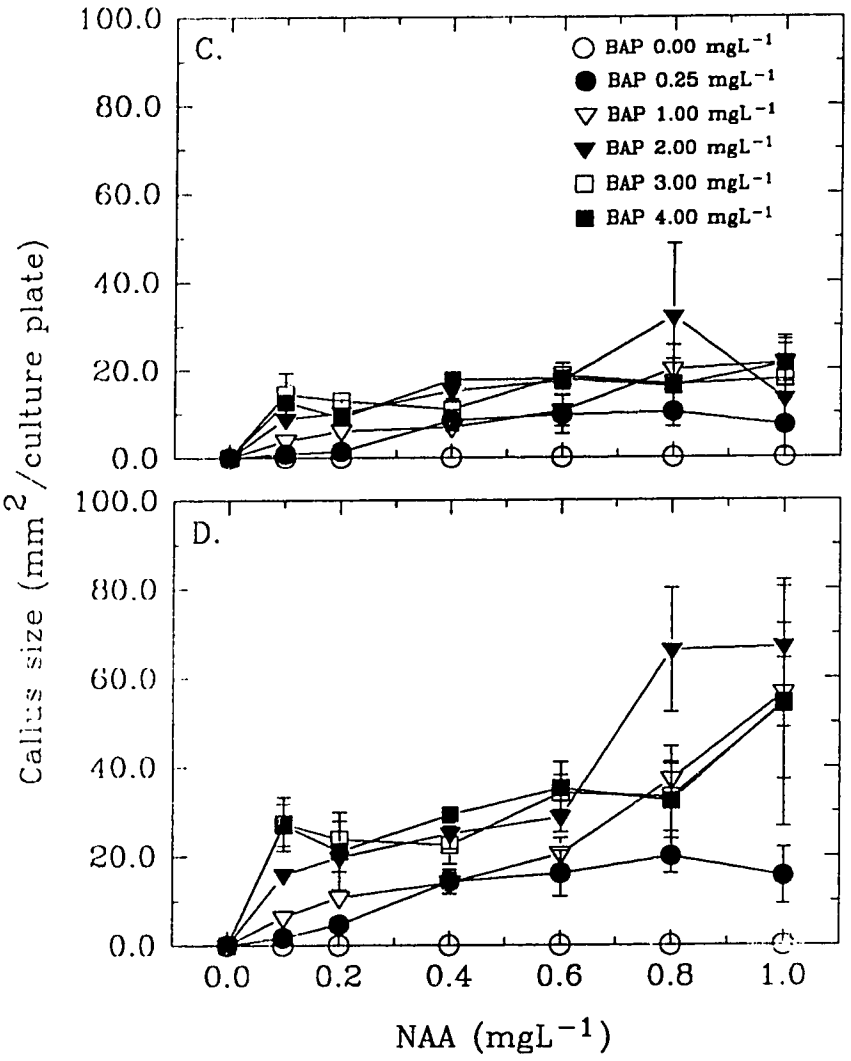

Fig. 4.3.1 (continued) The influence of a dose response series of the auxin naphthaleneacetic acid (NAA) and the cytokinin benylaminopurine (BAP) on callus induction and growth over an eight week incubation period. Hormones were supplied in a semisolid medium. (C) Callus size six weeks after inoculation, (D) Callus size eight weeks after inoculation.

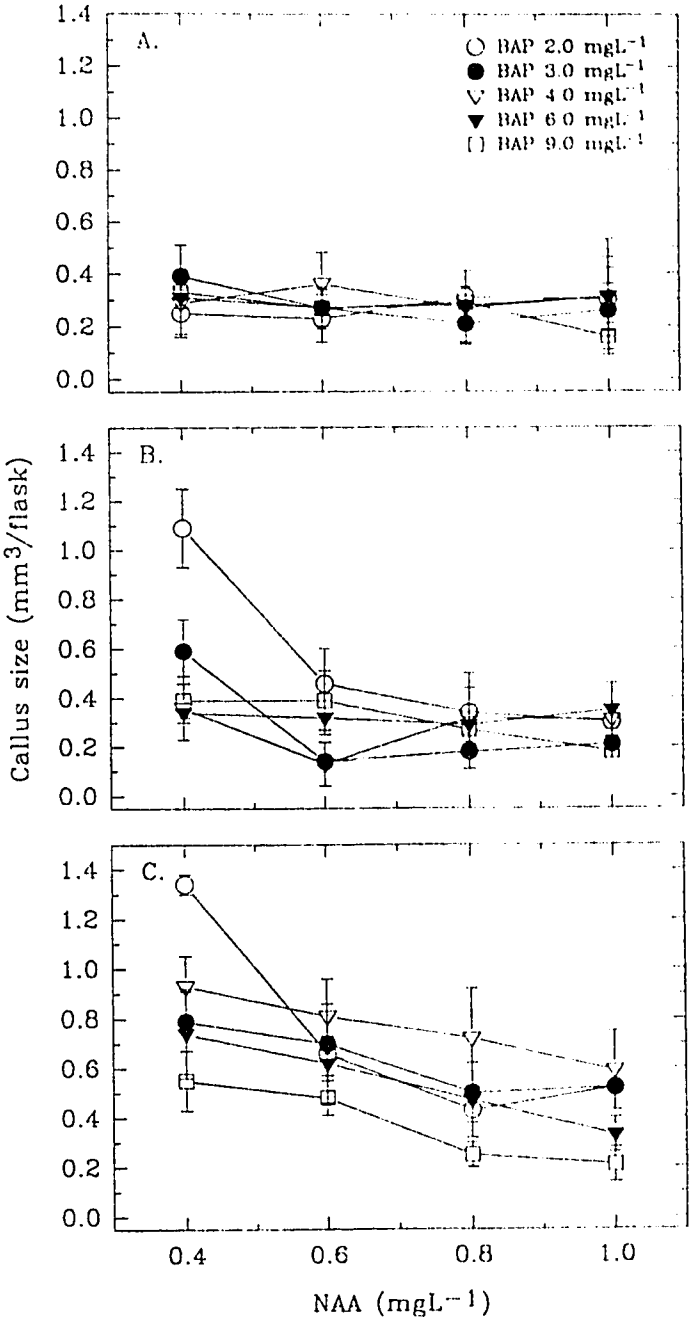

Fig. 4.3.2 Callus growth (± s.e.) measured in response to a selected range of auxin (NAA) and cytokinin (BAP) concentrations predetermined from the results of the initial callus induction experiment. Growth was maintained over three subcultures and measured as height x area (mm^3). (A) Callus size four weeks following initial subculture from induction experiment, (B) Callus size after the second subculture, (C) Callus size after the third subculture.

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5 General Discussion

Concern for reclamation of surface mining disturbances in Western Canada has increased in the last decade. Heightened environmental awareness of the late 1960's and the growth of surface mining during the 1970's have lead to the current status of reclamation in the resource industry of Alberta. Increasing interest and some initial success in using native shrubs for revegetation has lead to the testing of wild rose as a species for bioengineering. King *et al.* (1983) reported that although wild rose is potentially a valuable pioneer species, its current usefulness is limited due to the lack of large amounts of healthy plant material available. Therefore, the overall objective of this research was to develop methods for mass propagation of wild rose for reclamation by (1) breaking seed dormancy and enhancing germination, (2) rooting of stem cuttings and (3) producing callus through *In vitro* techniques that would later lead to organogenesis.

In chapter two, attempts to break dormancy and increase germination rates of wild rose provided a promising approach through the propagation of ripe achenes. While dual stratification treatments, *in vitro* germination, and methods of surface scarification by mechanical abrasion and acid digestion of the seed coat proved to be ineffective in promoting germination, cutting the achene in half transversely prompted rapid germination. Exposing the embryo in this way removed the mechanical restraint imposed by the seed coat as well as permitting rapid imbibition. Inhibitors which prevent growth and development of the embryo may also be released upon removal of the seed coat. As a result, cut ripe wild rose achenes exhibited cumulative germination of 82% in two weeks, which was four times greater than germination in any other treatment. The technique of nicking or cutting the seed coat of achenes has previously enhanced germination of other rosaceous plants such as strawberries (Miller *et al.*, 1992). Although cutting achenes to

promote wild rose germination appeared successful, the process was time consuming and labor intensive, which lead to research in other techniques for propagation.

Propagation of wild rose by stem cuttings was studied as a second technique. Rooting success was influenced by the status of the stem and concentration of the rooting hormone, but the rooting medium appeared to have little influence on root production. Furthermore, the results reflected the importance of foliage on root production. By defoliating stem cuttings, root production decreased dramatically. Exogenous IBA concentrations of 0.8% applied to wild rose softwood stem cuttings appeared to be the most effective treatment in promoting root formation. However, the current literature presents conflicting information with respect to rooting of softwood and hardwood stem cuttings. Hermesh and Cole (1983) have suggested that hardwood cuttings have a greater propensity to produce roots than softwood cuttings while Osbourne (1990) recommended the use of softwood cuttings. Therefore, unfortunately it appears that rooting of wild rose stem cuttings is not only subject to the influence of rooting hormones and medium but the environmental factors that determine the physiological status of the stock plant as well. I conclude, therefore, that propagating wild rose by stem cutting is unpredictable, and therefore requires further study.

With the propagation of wild rose by stem cuttings, one plant is produced for every stem cutting that roots, and when wild rose seeds germinate, each produces a single plant. In contrast, the goal of culturing explants by *in vitro* techniques is to obtain numerous cloned plants from the same donor tissue. With tissue culture it is possible to select and produce plants with desirable traits suitable for specific reclamation sites. The present experiment was undertaken to investigate the potential for callus production and subsequent organogenesis of wild rose embryos and leaf explants. An initial attempt to induce callus from embryos was spoiled by complete contamination of the cultures by

bacteria originating either within the seed coat or introduced during aseptic handling. Callus induction and growth from leaf explants required both auxin and cytokinin in the medium. Callus induction on leaf explants occurred at auxin levels of 0.8 to 1.0 mgL^{-1} NAA and cytokinin levels greater than 0.25 mgL^{-1} BAP. To enhance callus growth, optimum ranges of NAA and BAP were chosen and tested from the induction experiments. Throughout three successive subcultures, callus appeared to respond more effectively to a NAA concentration of 0.4 mgL^{-1} and BAP concentrations between 2.0 and 4.0 mgL^{-1}. With the establishment of a range of concentrations for NAA and BAP for callus induction and growth on wild rose leaf discs, the induction of organogenesis should be possible in the near future. It will however, be important to modify existing protocols and develop reliable regeneration system to encourage the production of wild rose plantlets. The use of tissue culture techniques has allowed the rates of multiplication of some plants, such as *Rubus* (Cousineau and Donnelly, 1991), to be increased to the extent that micropropagation *in vitro* has become economically advantageous.

These studies have demonstrated the possibilities of using both non-vegetative and vegetative methods to propagate large numbers of wild rose. Breaking dormancy of achenes by exposing the embryo, produces reliable and rapid germination. Through vegetative methods, the rooting of woody cuttings can also be successful with the appropriate treatments. Application of rooting hormones to foliated softwood wild rose stem cuttings successfully promotes the formation of roots. In addition to these traditional methods of propagation, micropropagation is becoming increasingly important for commercial propagation of roses because of its rapidity, relative cheapness and potential for planned production. Although the present study did not reach the stage of plantlet regeneration for wild rose, callus induction and growth became routine leaving open the possibility for organogenesis. Therefore, it is feasible to encourage further study in this area. At the same time, it is sensible to suggest that the difficulty of propagating wild rose

by in vitro techniques, dictates that micropropagation should not be the sole propagation method. Therefore, it is recommended that several propagation techniques such as breaking germination, rooting of stem cuttings and micropropagation be incorporated into the field of bioengineering for the purpose of propagating large numbers of wild rose for reclamation.

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6 Appendix

Appendix 6.1 Anova table for the number of roots produced and the root weights measured for propagating wild rose by stem cuttings. The model involves three rooting medium (sand, loam, and metro mix) and four IBA hormone levels (0.0, 0.1, 0.3 and 0.8%).

Number of Roots:				
Source	DF	Sum of Squares	F Values	Pr > F
Model	35	15471.67	27.83	0.0001
Error	288	4575.33		
Corrected Total	323	20047.00		
Root Weight:				
Source	DF	Sum of Squares	F Values	Pr > F
Model	35	5.2605	10.96	0.0001
Error	288	3.9513		
Corrected Total	323	9.2118		

Appendix 6.2 Murashige and Skoog modified basal medium stock solution supplemented with 6-benzylaminopurine and alpha-naphthaleneacetic acid.

Chemicals and Components	Concentrations (gL^{-1})
KNO_3	190
NH_4NO_3	165
$CuSO_4 \cdot 5H_2O$	0.0025
$MgSO_4 \cdot 7H_2O$	37.00
$MnSO_4 \cdot H_2O$	1.69
$ZnSO_4 \cdot 7H_2O$	0.86
$CaCl_2 \cdot 2H_2O$	44.00
$CoCl \cdot 6H_2O$	0.0025
KI	0.083
H_3BO_3	0.62
KH_2PO_4	17.00
$NaMoO_4 \cdot 2H_2O$	0.025
$FeSO_4 \cdot 7H_2O$	2.78
Na_2EDTA	3.72
glycine	0.20
myo-inositol	10.00
nicotinic acid	0.05
pyridoxine HCl	0.05
thiamine HCl	0.10
sucrose	30.00
Bacto-agar	8.00

Appendix 6.3 Duncan's Multiple Range test (alpha = 0.05) for callus initiation and growth in petri dishes in response to a range of concentrations of NAA (auxin) and BAP (cytokinin).

Time	[BAP]	N	Mean	Group	[NAA]	N	Mean	Group
1	1.00	24	1.44	A	1.00	20	2.32	A
	3.00	24	1.27	A	0.80	20	1.45	B
	4.00	24	1.25	A	0.60	20	1.28	BC
	2.00	24	1.04	A	0.40	20	0.78	BC
	0.25	24	0.72	A	0.10	20	0.60	BC
					0.20	20	0.45	C
2	4.00	24	6.94	A	0.80	20	6.05	A
	2.00	24	5.27	AB	0.60	20	6.05	A
	3.00	24	5.08	AB	0.40	20	5.53	A
	1.00	24	4.21	BC	1.00	20	5.05	AB
	0.25	24	2.44	C	0.20	20	3.53	B
					0.10	20	2.53	B
3	2.00	24	16.19	A	0.80	20	19.05	A
	4.00	24	15.90	A	1.00	20	16.25	AB
	3.00	24	15.25	A	0.60	20	14.9	AB
	1.00	24	11.50	AB	0.40	20	11.88	BC
	0.25	24	6.40	B	0.10	20	8.18	C
					0.20	20	8.03	C
4	2.00	24	37.10	A	1.00	20	49.63	A
	4.00	24	33.27	AB	0.80	20	37.80	AB
	3.00	24	32.54	AB	0.60	20	26.98	BC
	1.00	24	24.23	B	0.40	20	21.03	C
	0.25	24	12.10	C	0.20	20	16.03	C
					0.10	20	15.65	C

Appendix 6.4 (a) Duncan's Multiple Range tests (alpha = 0.05) for callus growth in Erylenmyer flasks over three subcultures. and in response to a range of concentrations of NAA (auxin) and BAP (cytokinin).

Subculture	N	Mean	Group
3	80	0.6183	A
2	80	0.3469	B
1	80	0.2839	B

Appendix 6.4 (b) Duncan's Multiple Range tests (alpha = 0.05) for callus growth in Erylenmyer flasks over three subcultures and in response to a range of concentrations of NAA (auxin) and BAP (cytokinin).

[BAP]	N	Mean	Group	[NAA]	N	Mean	Group
2.00	48	0.5202	A	0.40	60	0.5797	A
4.00	48	0.4508	AB	0.60	60	0.4072	B
3.00	48	0.4123	B	0.80	60	0.3418	B
6.00	48	0.3856	BC	1.00	60	0.3367	B
9.00	48	0.3127	C				