Ex situ CONSERVATION PROTOCOLS FOR THE RARE PLANTS *BRAYA LONGII* (ENDANGERED), *BRAYA FERNALDII* (THREATENED) (BRASSICACEAE) AND *SALIX JEJUNA* (ENDANGERED) (SALICACEAE) ENDEMIC TO THE LIMESTONE BARRENS OF NEWFOUNDLAND

JONI DRISCOLL

Ex situ Conservation Protocols for the Rare Plants Braya longii (endangered), Braya fernaldii (threatened) (Brassicaceae) and Salix jejuna (endangered) (Salicaceae) Endemic to the Limestone Barrens of Newfoundland

by
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Abstract

The Limestone Barrens of the Great Northern Peninsula of Newfoundland, Canada contains many rare plant species. The Great Northern Peninsula (GNP) contains 114 rare plant species; in fact this is the only place in Newfoundland and Labrador where 29 of these 114 rare plant species can be found (Bouchard et al., 1991). Part of this area is considered critical habitat (meaning it is necessary for the survival or recovery of a listed species) for three world endemics: Braya longii (endangered,) Braya fernaldii (threatened) (Brassicaceae), and Salix jejuna (endangered) (Salicaceae) (COSEWIC, 2005). These species are listed by The Committee on the Status of Endangered Wildlife in Canada (1997 and confirmed in 2000 for both Braya species; 2001 for Salix jejuna) as well as the Provincial Endangered Species Act (2002) and federal Species at Risk Act 2003. The goal of this study was to develop reliable *ex situ* protocols for the growth and maintenance of these three listed species. Both Braya species did not grow sustainably in captivity; therefore, through analysis of natural soil nutrient content and particle size, a reliable soil mix (1:1 mix of braya mix to crushed limestone) was produced that mimicked natural growth. Based on studies of other willow species Salix jejuna seed was assumed to have low longevity and viability which is why a main focus of developing an *ex situ* population of S. jejuna was on establishing a protocol for the survival of cuttings (Arya et al., 1988; Maroder et al., 2000). Not only was a successful protocol established for survival of S. jejuna cuttings, but the germination study provided evidence that the seed is viable for up to 9 months if stored in a freezer at -20^0C. In light of this a seed gene bank may be possible with S. jejuna seed.

The multiplication of both B. longii and B. fernaldii in tissue culture was unsuccessful; none of the plants survived longer than 1 month after transfer from test tube to soil (acclimatization). Possible explanations for this could include the inability to produce leaves, roots and a viable meristem in every explant. The use of tissue culture as a tool to multiply S. jejuna was very successful; survival rate was as high as 94.8% after acclimatization from test tube to soil. Recommendations for B. longii and B. fernaldii include maintaining, and supplementing when necessary, a long-term seed gene bank and the existing ex situ populations (in developed soil mix) and continuing long-term monitoring and protection. Recommendations for S. jejuna include maintaining, and supplementing when necessary, existing *ex situ* populations (by means of cuttings), establishing a long-term seed gene bank and continuing research, monitoring and protection of *in situ* populations.

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Co-Authorship Statement

All manuscripts in this thesis were co-authored with Luise Hermanutz and Wilf Nicholls. In all instances I was the principle contributor to project design and proposal, implementation of the field research component, analysis of the data and manuscript preparation.

Chapter 1

General Introduction

The number of endangered species around the globe in 2005 according to the International Union for the Conservation of Nature is 15, 589, with 8 321 of these being plants (IUCN Red List, 2005). The percentage of these endangered plant species that are currently part of an *ex situ* conservation program is unknown though suspected to be small; some estimate that seed storage accounts for the vast majority, around 90%, of those currently in an *ex situ* program. Target 8 in the Global Strategy for Plant Conservation suggests that 60% of the world's endangered plants be included in an *ex situ* conservation program and that 10% of them should be included in a recovery program by 2010 (BGCI, 2006). While this goal is extremely ambitious, Botanical Gardens Conservation International suggests that it is indeed possible considering the widespread availability of *ex situ* conservation facilities.

The Committee on the status of endangered wildlife in Canada (COSEWIC) lists 500 species at risk within Canada. 159 of these species are plants: 74 are endangered, 48 are threatened, 35 are of special concern and 2 are extirpated. COSEWIC status categories for listed species include: 1. Extinct, a wildlife species that no longer exists, 2. Extirpated, a wildlife species that no longer exists in Canada, but occurs elsewhere, 3. Endangered, a wildlife species facing imminent extirpation or extinction, 4. Threatened, a wildlife species likely to become endangered if limiting factors are not reversed and 5. Special Concern, a wildlife species that may become a threatened or endangered species

because of a combination of biological characteristics and identified threats (COSEWIC, 2005).

Study Species

The Strait of Belle Isle region in Newfoundland contains 114 rare plant species; in fact it is the only place in Newfoundland and Labrador where 29 of these 114 rare plant species can be found (Noel, 2000; Bouchard et al., 1991). Three of these species are endangered endemics. These plants are: Braya longii (endangered,) Braya fernaldii (threatened), and Salix jejuna (endangered) (COSEWIC, 2005). These species are listed under COSEWIC (1997 and confirmed in 2000 for both Braya species; 2001 for Salix jejuna) as well as the provincial Endangered Species Act (2002) and federal Species at Risk Act (2003).

Braya fernaldii and B. longii (Family Brassicaceae) are arctic/alpine-like perennials restricted to the Great Northern Peninsula (GNP) of Newfoundland. While the GNP is not considered part of arctic or alpine ecosystems the habitat is similar and therefore the flora is similar to arctic or alpine systems. Braya longii is restricted to a 10 km long coastal strip from Yankee Point to Shoal Cove (Figure 1.1) (Table 1.1). Braya fernaldii is restricted to a 120 km long coastal strip from Port aux Choix to Burnt Cape (Greene, 2002; Hermanutz et al., 2002). Within this area study sites have been set up for long-term monitoring (Figure 1.2). However, less than 10% of these coastal strips contain their preferred habitat: open, vegetation-free, disturbed substrate (Greene, 2002).

Salix jejuna (Barrens willow) is a small prostrate woody shrub with a very limited distribution. It is restricted to a 30 km long coastal strip from Watts Point Ecological Reserve to Cape Norman (Djan-Chékar et al., 2003). There are currently 8 study sites set up within this area for long term monitoring of S. jejuna (Figure 1.3). While S. jejuna can be found throughout its range the distribution is by no means continuous, therefore the sites were established after visual inspection in order to choose sites that contained enough plants to warrants a study site. Also, while relatively open and vegetation-free substrate is required for Salix jejuna, the link between frost-heave action and colonization is not as obvious as with the braya species.

Threats

The high level of threat faced by these plants, coupled with their low numbers and the restricted nature of the populations, makes them highly vulnerable to local extirpation. The primary reason all three limestone barrens species are currently under threat is due to anthropogenic disturbance, most notably land use activities such as development around communities and limestone quarrying (Janes, 1999). The fact that these plants are rare only increases their risk of extinction; it is not the prime reason for their current predicament. Another threat that currently affects both B. longii and B. fernaldii is herbivory by a non-native insect pest, the Diamond back moth (Plutella xylostella) (Hermanutz et al. 2005).

Strategies for plant conservation

The need for *in situ*, or on site conservation is paramount as seen from the sheer number of endangered species around the globe; we must strive to preserve species within their natural habitat (IUCN Red List, 2005; Simmons et al., 1976). Species conservation cannot be considered independent of habitats and ecosystems; in many cases protecting a species also protects valuable habitat as well as numerous other species that thrive within it (Given, 1994). However, while the best solution to preserving an endangered species is by habitat preservation, it is not always an option. Many habitats have been irreparably damaged by human activities, making on site conservation practically impossible. Even if habitat conservation is possible it quite often requires the reintroduction of plants in order to increase the population sizes to a more stable level (Maxted et al., 1997). While *in situ* conservation is critical for endangered species, the importance of *ex situ* (or off-site) conservation is equally vital. Without an *ex situ* population as a "back-up" or failsafe, the natural populations may be very vulnerable, in fact a single natural or anthropogenic disaster might cause the extinction of an entire species, such as narrowly distributed endemics.

Ex situ conservation is the conservation of plants away from their natural area of occurrence, and can include living collections of plants, gene banks, clonal collections, germplasm banks, seed gene banks, pollen banks and preserving plants in tissue culture (Given, 1994). The importance of *ex situ* conservation in the management and protection of an endangered plant is important for several reasons: first, an *ex situ* population will be

invaluable as a failsafe in the event that the natural populations are extirpated; second, it can supply the plant material needed for reintroduction; and third, it can also be used for experimentation to reduce impact on natural populations. *Ex situ* populations can arrest the erosion of the genetic diversity that may be occurring in nature, giving species a long-term chance of survival (Given, 1997; Heywood, 1990). Unfortunately there are a few disadvantages associated with *ex situ* conservation. *Ex situ* conservation can be expensive to establish and maintain and it is difficult to capture the range of genetic variation required to emulate the natural populations. Because of this, *ex situ* conservation is most effective when done concurrently with on site conservation (Given, 1997). The Convention on Biological Diversity also states that there is a vital role *ex situ* research should take to compliment these *in situ* measures (Maunder, 1994). Three major *ex situ* strategies that are the focus of this research include persistence of live plants, seed gene banking and tissue culture.

Persistence of live plants

Several methods are used in *ex situ* conservation to establish viable populations and maintain the genetic diversity of a species (Given, 1997). An important method is the development of protocols for the persistence of live plants. While much work has been done in the horticultural industry on the development of such protocols for arctic/alpine ornamental plants (Kuzovkina and Quigley, 2004; Cheong et al., 1999 and Kim et al., 1996), this is one of the first studies to focus on growing endangered arctic/alpine-like plants. Experimentation with substrate characteristics, drainage and

environmental conditions are all necessary to determine the growing conditions that will mimic natural conditions to ensure natural growth and reproduction of these plants. Unlike the goal of the horticulturalist, which is to optimise or maximize the growth of plants, the ideal growing conditions for these endangered plants should produce natural and sustained growth. Previous attempts to grow an *ex situ* population of both Braya species were not successful; all plants grew too rapidly with the majority dying within a year and the remainder within 3 years. Therefore knowledge of the substrate characteristics, drainage and environmental conditions necessary for sustained growth is crucial to successful *ex situ* strategies.

Seed Gene Banking

Another method that is employed in *ex situ* conservation is the establishment of a seed gene bank. A seed gene bank is a collection of seeds from wild plant species kept in long-term storage, usually at -20^{0}C and/or low humidity. Many countries have large seed gene banking facilities. Two examples are The Wellcome Trust Millennium Building at KEW Botanical Garden (Royal Botanical Garden, 2005) and the National Seed Bank of Canada (Saskatoon).

While both Braya longii and B. fernaldii seeds remain viable for several years if stored in a freezer at -20^{0}C (Hermanutz and Parsons, 2002), it was assumed that, as is common in other willow species (Arya et al., 1988 and Maroder et al., 2000), Salix jejuna seed would have low longevity and viability. The ability or potential to successfully bank S. jejuna seed will play a critical role in the ability to develop and maintain an *ex*

situ population that preserves individuals from its entire distribution. Seed gene banking, while important, is not in itself a complete solution. The benefit of storing seed for a long period of time is lost if there aren't the protocols developed to test viability while in storage or successfully grow out the plants when they are needed for either maintaining an *ex situ* population or for reintroduction.

Tissue culture

The development of tissue culture protocols for the use in endangered species conservation is becoming more common, though still not widely used (Liao et al., 2004; Kyte and Kleyn, 1996). This is for several reasons; first it allows plants to grow in a sterile environment, free of bacteria, fungus and insects. Second, it allows for the mass propagation of individuals from a very small amount of tissue, whether it be shoot tips, seeds, roots or nodal cuttings. Third, tissue culture allows for clonal multiplication, which can be advantageous for the selection of desired genotypes for experimental purposes. While tissue culture may seem to be a solution to the problem of endangered plants it is important to note that if plants cannot be successfully transplanted from test tubes to either pots or the natural sites then it is an ineffective method of preserving the species (Liao et al., 2004). Any new tissue culture protocols on endangered plants should focus on the survival of plants from test tubes to soil.

Previous *In situ* Research

Both Braya species have been studied *in situ* since 1998. Presently, several concurrent studies are taking place regarding their *in situ* status. As of 2005 8 years of long-term data have been gathered on these species; demographic information on the plants life cycle, size, reproductive output and damage by insects has been investigated. These data will be used at a later date to conduct a Population Viability Analysis (PVA). (Tilley and Hermanutz, 2005; unpublished data).

In addition to the long-term studies, the impacts of a non-native insect pest, the Diamond back moth (Plutella xylostella), on braya survival is being investigated (Tilley, 2005 unpublished data). The Diamond back moth (DBM) is an invasive agricultural pest that annually gets blown into Newfoundland by wind currents. It is a pest for all Brassicaceae crops including broccoli and cabbage, and will also feed on both braya species. The impact on these species is significant: 25% of all braya over the past 3 years have been infested with the DBM. Therefore understanding this threat is vital to the persistence of the *in situ* populations of both B. longii and B. fernaldii (Tilley, 2005 Unpublished data).

Salix jejuna had not been studied in any extensive detail before this project began in 2003. A brief investigation of plant occurrence, as well as some tagging and measuring had been done in order to prepare the recovery strategy for the barren's willow (Djan-Chékar et al., 2003). Therefore, the focus for this project, with respect to S. jejuna,

has been on gathering base-line data and starting up long-term monitoring for future research endeavours.

Previous *Ex situ* Research

In 2001 and 2002 several attempts to grow both braya species at the Memorial Botanical Garden had been met with limited success. Germination was very successful but unfortunately the plants grew too rapidly, reproduced much sooner than in nature and died within 3 years. These plants completed their life cycle more quickly than in nature which most likely left them weakened and vulnerable to pests and diseases.

There had not been any extensive attempts to grow *ex situ* populations of S. jejuna. This project, therefore, focused on gathering preliminary germination and vegetative propagation data, including cutting survival and seed germination and longevity.

Research Objectives

The goal of this study was to develop reliable *ex situ* protocols for the growth and maintenance of Braya longii, B. fernaldii and Salix jejuna, as this is a recovery objective for all three species (Appendix 1). These goals included first collecting life history data on S. jejuna, including population density, size of plants and reproductive potential. This information helped to direct this study and will be used for future research. Second, soil mixes were developed, in an attempt to facilitate a more natural, slow and sustained growth for both braya species; since establishing and maintaining populations of live

plants is invaluable in *ex situ* conservation. These soil mixes would be based on chemical and textural analysis of the natural substrate. Third, the viability and longevity of S. jejuna seed was to be evaluated. The optimal storage conditions for sustained longevity of S. jejuna seed, i.e. drying, freezing or a combination of both was tested for the purpose of seed gene banking potential. Fourth, the viability of tissue culture as a technique for mass propagation of these species was tested. Particular importance was placed on the acclimatization stage of all three species from tissue culture to soil, as the future benefit from this work would be the possibility of reintroduction. It was felt that a comparison of these species, with respect to which *ex situ* strategies are most effective, i.e. persistence of live plants, seed gene banking or propagation in tissue culture, would greatly contribute to our understanding of *ex situ* conservation with regards to endangered arctic/alpine like plants.

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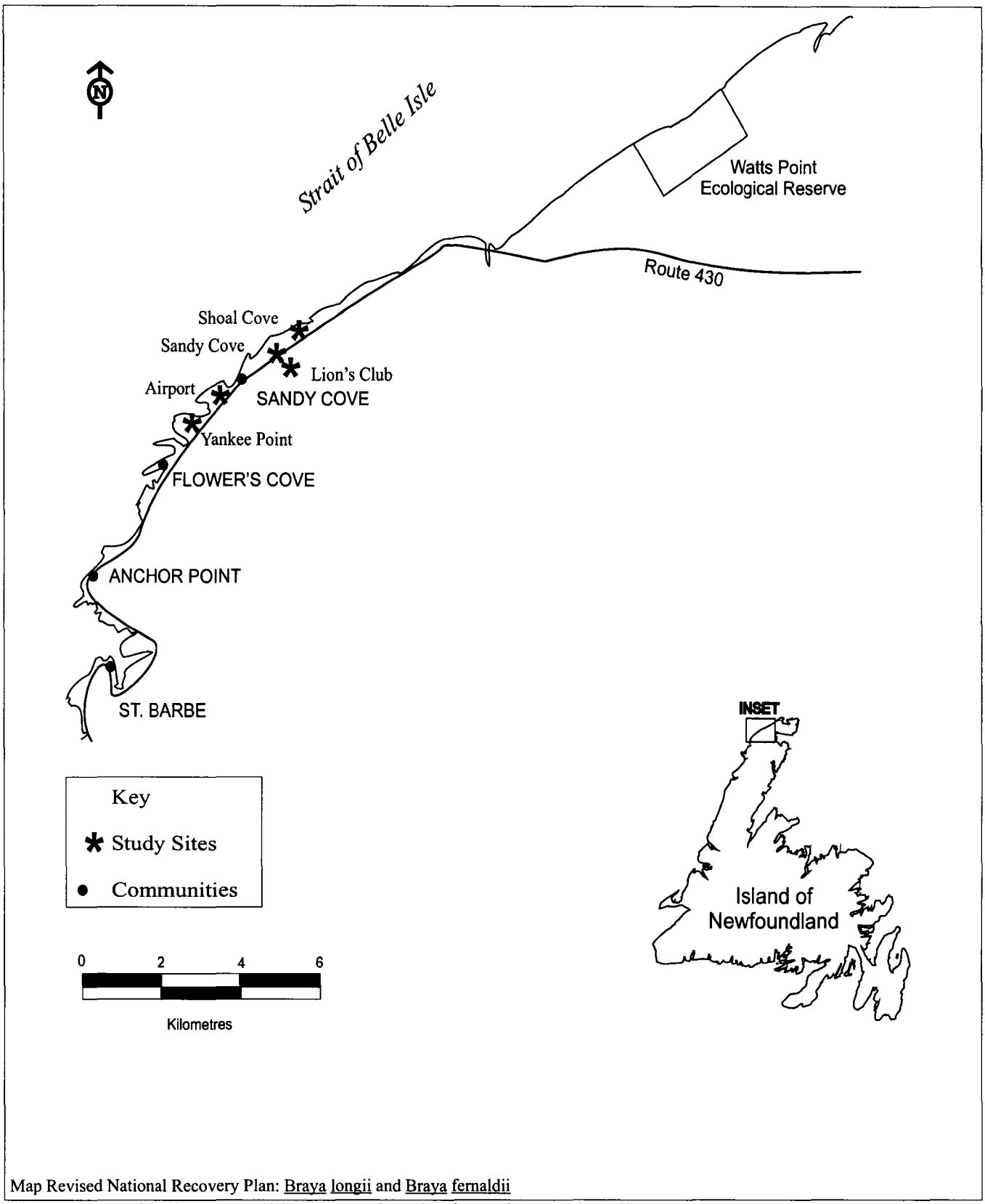
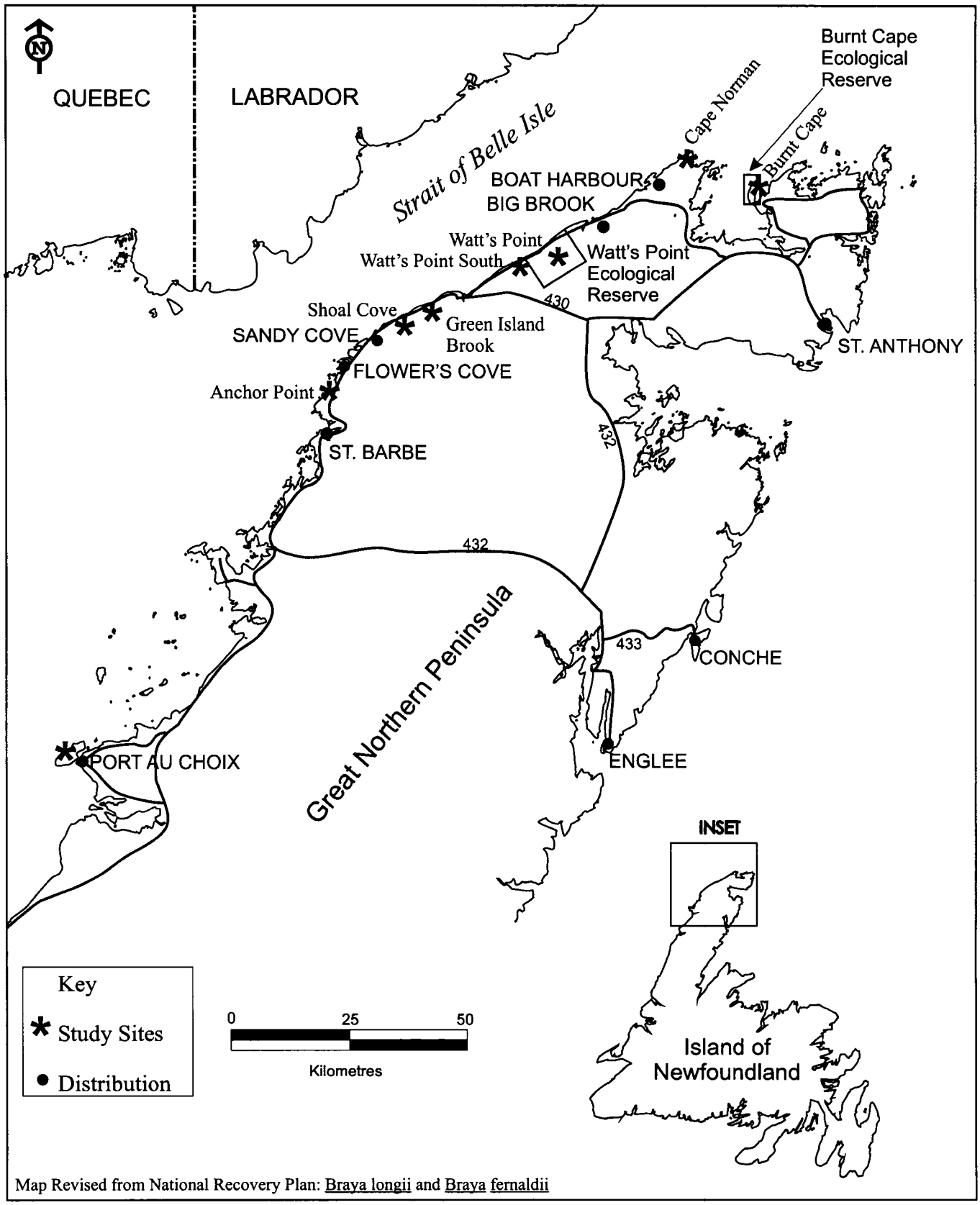
Table 1.1: Population size of known populations of Braya longii (BL) and B. fernaldii (BF) on the Great Northern Peninsula, Newfoundland. Population size refers to flowering individuals (pre 2005 data adapted from Hermanutz and Parsons (2002) and 2005 data from Tilley (2005 unpublished data).

Species	**Site and Disturbance**	**Most recent population count**	**Population size**
BL	Airstrip Disturbed (APD)	1998	2400
BL	Airstrip natural (APN)	1998	900
BL	Lion's Club Disturbed (LCD)	2005	355
BL	Lion's Club Natural (LCN)	2005	8
BL	Sandy Cove Disturbed (SCD)	2005	178
BL	Sandy Cove Natural (SCN)	2005	260
BL	Shoal Cove Disturbed (ShoCoD)	2005	230
BL	Yankee Point Disturbed (YPD)	2004	3529
BL	Yankee Point Natural (YPN)	2005	4
BF	Anchor Point East Natural (ANC)	2000	250
BF	Anchor Point West/St. Barb Natural (St. Barb)	2004	167
BF	Burnt Cape Disturbed (BCD)	1998	850
BF	Cape Norman Natural (CNN)	2005	44
BF	Green Island Brook Disturbed (GIB)	2005	404
BF	Port aux Choix Natural (PAC)	2005	98
BF	Shoal Cove Natural (ShoCoN)	2005	4
BF	Watt's Point Disturbed (WPD)	1998	800



Map Revised National Recovery Plan: Braya longii and Braya fernaldii

Figure 1.1: Distribution and location of study sites of Braya longii.



Map Revised from National Recovery Plan: Braya longii and Braya fernaldii

Figure 1.2: Distribution and location of study sites of Braya fernaldii on the Great Northern Peninsula.

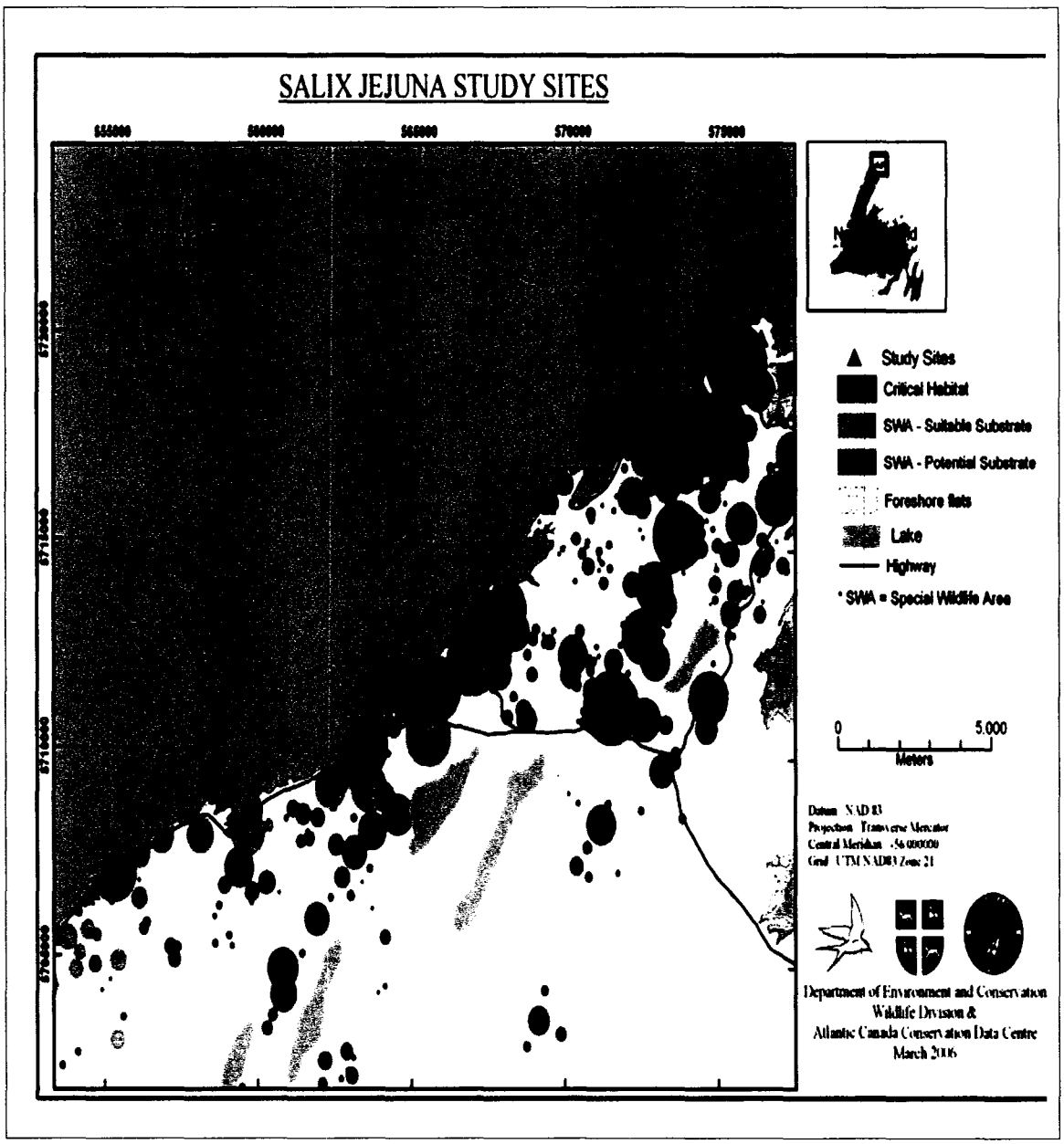


Figure 1.3: Distribution and location of study sites of Salix jejuna on the Great Northern Peninsula.

Chapter 2

Preliminary Investigation of Salix jejuna Life History

Life history information, such as population density, growth rate, and reproductive capacity were studied at all 8 currently established S. jejuna sites in order to better understand the endangered status of this species.

Clonal Growth

Many plants, being more plastic than animals, have the ability to reproduce sexually and asexually. A form of asexual growth that can be very advantageous is clonality, where a plant genet will invest its resources to produce genetically identical off-shoots (called ramets) instead of, or in addition to, seeds. A model of reproduction in clonal plants by Gardner and Mangel (1999), suggests that high rates of clonal propagation are advantageous for a species. However, as in many similar cases, populations with high rates of clonal propagation have low rates of seedling establishment. Despite the apparent advantage of clonal propagation in maintaining populations, the importance of seedling establishment is not undermined, in fact it can be vitally important in maintaining the genetic diversity of a population and thereby the success of an entire species as a whole.

Understanding the clonal nature of a species, as stated in Barsoum (2002) helps one understand the genetic diversity of a population and what the effective population size needed for protection might be, the implications of the sexual vs asexual

investments, and how demographic pressures can effect the evolutionary change of a species.

Many willows have the ability to grow clonally, by means of underground stems; this makes it more difficult to determine genetically different individuals (Lian, et al., 2003; Rottenberg, et al., 1999). The clonal nature of Salix species is widely noted and presents a problem to most *in situ* studies (Tamura and Kudo, 2000; Douglas, 1995) which makes tagging and collecting tissue (whether it is cuttings, seeds, leaves etc.) from distinct individuals very difficult. While a plant may look distinct and not connected to any surrounding members of its species it is difficult to tell unless it is either excavated to determine interconnections, or analyzed genetically. To minimize the probability of collecting ramets rather than genets many studies have simply selected plants as far apart as possible and worked on the assumption that they are distinct individuals (Tamura and Kudo, 2000).

Population density

In populations of plants that may exhibit clonal growth, direct population counts are not always the best method to use because of the necessary assumption that plants are distinct individuals (Tamura and Kudo, 2000). If there is no way of determining if one seemingly distinct plant is connected underground to other plants then a direct count may count the same individual more than once (Tamura and Kudo, 2000). For this reason, population density (by means of line transects) is often used and widely accepted in the place of population counts (Crain, 1998), especially where clonal growth is suspected.

While it is true that population density measured in this manner may be effected simply by growth rather than an actually population increase, it is suitable for monitoring the changes in population size of a species.

Sex Ratios

Populations of dioecious plants are expected to have a 1:1 sex ratio (Carvalho et al., 1998). However, this is quite often not the case in willows as the sex determination and sex ratios can be complex. In willows the sex ratio is often female biased; in a study on 5 arctic willows, including S. arctica and S. glauca, Crawford and Balfour (1990) showed a 60:40 female to male sex ratio. Some reasons for this have included differences in the rate of growth and mortality, herbivory and predation and allocation patterns between the sexes (Rottenberg et al., 1999).

Pollination of Arctic/ Alpine Plants

Arctic plants are generally thought to be independent of insects for pollination, but this assumption is often incorrect (Kevan, 1972). While it is generally accepted that willow species exhibit both anemophily (wind pollination) and entomophily (insect pollination), there is still much debate about which is the more common and important method of pollination (Kevan, 1972). Either limiting the pollen load or increasing it, by means of controlled pollination experiments can test the importance of entomophily in a given species. Hand pollination can increase the pollen load onto a catkin and mimic what would happen in a natural situation with insect visitation. If seed set changes with

an increase in pollen load than it suggests that wind pollination on its own does not deliver enough pollen to maximize the seed set and the plants are partially dependent on insect pollination (Fox, 1992).

Pollination of willows

Several studies on willows have tested seed set during pollination experiments and have shown that many arctic/alpine species (Peeters and Totland, 1999; Fox, 1992) as well as some temperate species are at least partially wind pollinated, if not mainly wind pollinated (Tamura and Kudo, 2000). As stated in Fox (1992) the seed set for an arctic Salix can increase significantly when hand pollinated. While these studies showed an increased seed set when catkins were hand pollinated (indicating pollen limitation) they did not show an increase in seed set between the insect inclusion and exclusion treatments, which might be expected if the plants were mainly insect pollinated (entomophilous).

Other flower traits, besides seed set that are thought to be important when determining the pollination system of willow species are nectar production, catkin colour, size and presentation, flower number and flower size (Peeters and Totland, 1999). Nectar, naturally, is an insect attractant so if a flower produces nectar it may be at least partially dependent on insect pollination. It would also be advantageous to have larger catkins and flowers if a plant was relying on insects for pollination. While in general many arctic plants have nectar and pollen producing, showy flowers, they may have little or no need for insects (Chernov, 1966; Kevan, 1972).

Objectives

In order to establish a viable *ex situ* population of S. jejuna that closely resembles the natural populations, diagnostic characteristics (population density, average plant size, number of catkins per plant and sex ratio) needed to be determined. A better understanding of how the plants were growing in the wild helped to determine the best way to grow them off site.

Materials and Methods

Salix jejuna study sites

While the approximate distribution of S. jejuna was known (Anions, 2000; Djan-Chékar et al., 2003), long-term study sites had not yet been established. With the aid of suitable habitat topographical maps provided by the Wildlife Division, eight sites were selected and are distributed throughout the natural range (Table 2.1). All sites are within the known distribution of S. jejuna and contained suitable habitat as well as a high enough population density to make it worthwhile for long-term monitoring, as many areas within the habitat contain S. jejuna but not in a great abundance. All sites are on undisturbed habitat with the exception of the CND site, which is on anthropogenically-disturbed habitat.

Tagging and Long-term monitoring of *in situ* Salix jejuna

At each of the eight S. jejuna study sites 20 individuals were tagged for long-term monitoring. Originally the tags were small pieces of aluminum and coloured flagging tape that were stuck into the soil by a nail. However, though the initial tagging was done in June 2004 several sites needed to be re-tagging because birds, presumably attracted to the shinny tags, pulled many of them out. To prevent the further removal by birds large headed nails replaced all the tags. A number, corresponding to the plant identification number was painted on top on each nail and a detailed map of the site was drawn in order to relocate the plants.

The sex of each tagged plant was recorded as well as the number of catkins in both 2004 and 2005. Due to the different developmental rates of male and female flowers the catkins on the males were counted in July (when the catkins were still underdeveloped) and females were counted in early August (before the ovaries had begun to swell). Digital pictures of all tagged individuals were taken and the diameter was measured using UTHSCSA image tool software.

Population density

In order to measure the density of S. jejuna plants, two line transects per site were established at each of the 8 established sites. These transects were 15 m in length and were placed approximately in the middle of each defined site. For each meter the number of times a stem or root crossed under the line was recorded. This variable was averaged per transect and per site. The density of S. jejuna at each site was recorded both in 2004 and in 2005 to determine if there is any change in population density on a yearly basis, as this may be a sign of a population increase or decrease.

Pollination of Salix jejuna

In order to determine whether fertilization was possible for S. jejuna a pollination experiment was conducted with *ex situ* plants. An enclosure made of Remay® cloth (spun nylon) was placed over all female and about half of the male S. jejuna plants contained in the off site population. This enclosure allowed air circulation within while excluding external sources of pollen. This ensured that female catkins would only be in

contact with pollen of the same species. While the enclosure cuts down on wind speed the objective of this experiment was not to emulate the natural *in situ* conditions, but rather to determine if fertilization was possible within the enclosure.

All available immature female catkins were allocated to 1 of 3 treatments. The first treatment was a control in which immature female catkins were bagged with individual Remay® cloth sacks and left sealed throughout the summer. If the cloth truly excluded all pollen then these control catkins would not be fertilized and would not produce any seed.

The second treatment tested the effectiveness of hand pollination. Immature female catkins were bagged with individual Remay® cloth sacks and every few days while the stigmas were receptive, mature S. jejuna pollen was brushed onto them. Several mature male catkins were used each day; each male catkin was brushed against the stigmas of 2-3 female catkins. The bags were re-sealed after hand pollination and remained that way until all stigmas were no longer receptive and the ovaries began to swell indicating their fertilization (approximately 1-2 weeks). They were then left un-bagged until the seed dispersal stage.

In the third trial female catkins, while inside the large cloth enclosure, were left un-bagged. They were left to naturally pollinate within the enclosure, in this way determining if natural wind pollination could be accomplished within it.

The maturing and swelling of the ovaries was noted throughout the summer. When the female catkins were highly developed, but not yet dehisced, cloth bags were tied around them. When the catkins started to dehisce all the released seeds were trapped

within the bag and could be counted and retained. The date of dehiscence, number of ovaries and seed set was recorded.

All catkins were allowed to naturally dehisce before seed was collected. An ovary was considered dehisced when it was cracked open, exposing the pappus of the seeds, which easily fell out of the ovary. Any seeds that required pulling to get out of the ovary were considered immature and were not used in this study. All the seeds were tested immediately for germination success. In all cases germination was considered successful when the radicle protruded from the transparent seed coat making the embryo bend into a "J" shape.

On Site Seed Germination

Seed was collected in 2004 for an *in situ* germination study in order to determine if seed could germinate in the natural environment, rather than just in soil. Seed was collected from the following S. jejuna study sites: CNA, CNB, BK1A and BK1B. These were the only sites used because they had sufficient available seed. As per the guidelines on the practice of seed collection from FloraBank in Australia (2006), no more than 20% of the fruit from any one plant is removed so as not to further endanger the wild populations. Several catkins were collected from each site and allowed to naturally open in a dry Petri dish. The released seed was collected and the pappus was removed to allow easier handling for planting.

Seeds were planted into a grid formation using a 0.5m square quadrat with intersecting lines. Twenty-five seeds per quadrat were sown onto the soil in this grid

formation and gently pushed into the substrate to prevent loss in this windy habitat. The top and bottom corners of the grid were permanently tagged so that the quadrat could be placed in the same position every time the seedlings were monitored. A brief sketch of the vegetation, most importantly the presence of any existing S. jejuna plants, was made for each of the quadrates in order to properly orient them in subsequent trips and to determine what growth was old vs what was new.

Each of the 4 sites included in this study had one quadrat sown with seed, with the exception of CNB, which had enough available seed to have two quadrats sown with seed within its site. Controls, which did not have any seed sown in them, were placed on all sites. Detailed sketches of the vegetation cover and absence/presence of S. jejuna plants and seedlings was taken for all 8 sites using the 0.5 m quadrat in order to monitor natural seedling establishment.

Data Analyses

Minitab® Statistical Software for Windows, Version 13.30 was used to carry out all statistical tests performed in this study. The General Linear Model (GLM) was used to carry out all statistical tests. For each test residuals were examined for normality, independence, and homogeneity to ensure that the assumptions of statistical tests were not violated. If the data were found to be non-normal then it was randomized as randomization tests make no assumptions (Schneider, 2003). However, if the sample size and the F-value were large, than randomization was not done, as the General Linear Model is known to be robust to violations of assumptions (Schneider, 2003).

P-values from these tests were used to determine whether differences between data were statistically significant when alpha was 5%. Minitab was used to carry out general descriptive statistics.

Results

Life History Data

The population density was determined using line transects. The descriptive variables included 'site', 'transect' and 'year'. The residuals were normal but there was a year*site interaction, therefore both years were tested separately. However, while the years were tested separately the significant interaction term implies that while there was a change in population density from 2004 to 2005, the sites did not all react the same way (Figure 2.1). The population density was significantly different among sites both in 2004 ($F_{[7,239]}$=106.13 $p<0.001$) and in 2005 ($F_{[7,239]}$ =110.52 $p<0.001$) (Figure 2.1). The site with the highest density of plants was BK1B, followed by BK1A. While it appears that the population density at CND was zero, it is not the case (Figure 2.1). There are plants at this site but the lack of stem crossings reflects the low density found at this site.

The diameter of the plants on site was analyzed with 'site', 'sex' and 'year' as descriptive variables. The residuals were normal and there were no significant interaction terms. The size of the plants (diameter) was not affected by the sex of the plant, therefore male and female plants are a similar size. The year was not significant, meaning there was no detectable growth from 2004 to 2005. While the amount of growth over 1 year was not detectable with the employed measurement techniques, it does not mean that the plants do not grow, but the amount they grow may not be measurable on a yearly basis, or the precision was not great enough to pick up *in situ* growth differences. The diameter of the plants is significantly different among sites ($F_{[7,373]}$ =13.48 $p<0.001$)

(Figure 2.2). Among the sites with the largest diameter is CND the only disturbed site. The plants had an average diameter of 20.7 cm at this site. The Big Brook site BK39 had the smallest plants with a diameter of 8.6 cm.

The number of catkins per plant will influence the reproductive output of a population. 'Site', 'sex' and 'year' were used as descriptive variables. The residuals were normal and there were no significant interaction terms. Year was not a significant factor so the 2004 and 2005 data were grouped. The sex of the plants did not significantly affect the number of catkins per plant. However, the number of catkins per plant was significantly different among sites ($F_{[7,477]} = 12.88$ $p<0.001$) (Figure 2.3). The CND site had the greatest number of catkins per plant (at over 22 catkins) and BK39 had the smallest number of catkins, at less than 1 per plant. These sites appear to be opposites, CND has the largest plants with the most catkins and BK39 has the smallest plants with the lowest number of catkins.

The proportion of male to female plants (the sex ratio) was tested. 'Site' and 'year' were descriptive variables; the residuals were normal and there were no significant interaction terms. The variable year was not significant. However, the proportion of male to female plants (the sex ratio) was different among the sites ($F_{[7,388]} = 5.29$ $p<0.001$) (Figure 2.4). While the sex ratio is different among sites it is not consistent, some sites have a higher proportion of males and the others have a higher proportion of females.

Pollination of Salix jejuna

Three treatments were used in this study; control catkins that were bagged before emergence, catkins that were hand pollinated and then bagged and catkins that were left open to be wind pollinated. None of the control catkins developed indicating that the enclosures did exclude all sources of pollen and apomixis is not present; therefore the control group was unavailable for statistical analysis. However, the germination success of seed from wind-pollinated catkins (catkins un-bagged but within the enclosure) was compared to the germination success of hand-pollinated catkins. The proportion of seed that germinated was not significantly different between the wind-pollinated and hand-pollinated catkins. Hand-pollinated catkins produced seed with an 81% overall germination rate, whereas wind pollinated catkins produced seed with an 82% overall germination rate (as these percentages are overall germination rates no standard error could be calculated).

Phenology of Salix jejuna

The basic phenology of Salix jejuna was monitored for *in situ* plants in 2004. As seen in many willow species the male S. jejuna plants started to develop their catkins earlier than the females. The flowering dates between females and males overlapped for a considerable amount of time in 2004 allowing time for pollination (Table 2.2). The female plants start to release seed around the 1st week of August and continue well into September but were spent by early October.

In situ seed addition plots

In total only 3.2% (4 of 125) of seeds planted on site at 4 sites in 2004 germinated. In the fall of 2004 one seedling was observed at CNB, one at BK1A, two at BK1B and none at CNA. In the summer of 2005 the plots were rechecked: two of the 4 from 2004 died and only 1 new seed germinated within the plots in 2005. The one seedling at CNB site had died but a new seedling was observed, the one seedling at BK1A died, the two at BK1B survived but no new seedling were observed and there was still no germination at the CNA site.

Discussion

This study indicates that population density, plant size and reproductive potential are not consistent between all currently established Salix jejuna sites. The density of plants among sites was dramatically different. The Big Brook sites (BK1A and BK1B) had the highest population densities, which is why protecting these sites is critical to the survival of the species. These sites are located down a road that is no longer maintained as the Big Brook community was recently resettled so the difficult access to these sites may actually prove to be an advantage from the perspective of protection.

The population density of the CND site (the only currently tagged disturbed site) was very low (Figure 2.1). It is then interesting to note that the plants at this site, while sparse, were the largest with the highest number of catkins per plant. Unfortunately, very few catkins at this site developed seed, in fact there was such a lack of seed that this site could not be included in the large germination and seed viability experiment. As this is an anthropogenically disturbed site, the plants could be of hybrid origin, which may explain the lack of fertilized seed. Willows are known to hybridize readily (Arnold et al., 1999) and because they are growing in a disturbed site they may have more potential to hybridize with other willow species. While the possible species S. jejuna could hybridize with on the GNP are many (S. reticulata, S. uva-ursi, S. caliculata, S. vestita, S. candida, S. glauca and S. arctophila) (Djan-Chékar et al., 2003) genetic analysis would have to be done to determine the possible parent species.

The importance of the CNB site should not be overlooked because of its low population density (Figure 2.1). Despite the low density the plants are very large, have a large number of catkins per plant and produce large quantities of seed. The habitat at this site is different from the other sites, with very little open exposed substrate patches, and most of the site is dominated by bedrock. The plants at this site grow between the cracks in the rocks, yet despite this, seem to grow very well. However, the lack of open substrate may present a problem for seedling recruitment. As determined from this study there is a lack of seedling recruitment at all sites, this may be connected with the abundance of bedrock at certain sites such as CNB.

Natural seedling establishment

Despite the fact that the germination success of S. jejuna seed is very high (see Chapter 3) the germination of seed on site was low; only 5 seeds of 125 (4%) in total germinated either in the planting year, 2004 or the subsequent year, 2005. Control plots were also established at each of the sites involved to determine if natural seedling establishment was occurring. No seedling germination was noted in the control plots in 2004 or 2005. However there were a few plants that appeared to be wild juveniles (due to the presence of woody stems). These juveniles were both in the control plots and the surrounding site and were very small with only 2-3 leaves each.

A study on willow seedling establishment by Gage and Cooper (2005) showed as high as 85-99% germination rate of willow seeds on site. And a study on Purshia subintegra, an endangered limestone plant had up to 58% germination on site

(Maschinski et al. 2004). Another study done on 4 Salicaceae species showed that not only was germination successful under laboratory conditions but it was also successful on site (Krasny et al., 1988). Depending on the soil surface seed for all four species (including one Salix species) germinated between 0% (at a very dry site) and 60% (Krasny et al., 1988). In a very similar experiment on the unrelated but endangered limestone species Braya longii and Braya fernaldii, the percent emergence *in situ* within seeded plots was between 50% and 94% (Tilley, 2003).

Why the S. jejuna seeds germinated in abundance under laboratory conditions (refer to Chapter 3) and not in the natural sites remain unanswered. Salix jejuna seeds germinated equally well on soil vs the Petri dish in the germination study; despite this seeds did not establish on site. Salix jejuna clearly does not lack viability, longevity or seed quantity, yet the relatively unsuccessful attempt at establishing seeded plots would suggest that there is a problem with either germination or establishment on site. Salix jejuna seed has a pappus and is wind dispersed; all naturally available seed may be dispersing too far away from suitable substrate to germinate. Any seed that remains on site may land on bedrock, which is not suitable for germination either. Despite this, it is still unknown as to why the seed sown on suitable substrate did not germinate.

This low rate of seedling establishment is common in clonal plant populations (Gardner and Mangel, 1999). The clonal nature of S. jejuna requires further study, however clonal growth within the family Salicaceae is very common and may partially explain the lack of seedling establishment (Tamura and Kudo, 2000; Douglas, 1995). Species that heavily rely on clonal growth do not invest a tremendous amount of

resources to seedling establishment. However, the importance of seedling establishment is not undermined, in fact it can be vitally important in maintaining the genetic diversity of a population and thereby the success of an entire species on the whole (Gardner and Mangel, 1999). Seedling establishment of S. jejuna will increase the genetic diversity within the populations.

Recommendations

All currently established S. jejuna sites should continue to be monitored in order to determine if the populations are stable. Salix jejuna is a long-lived plant therefore multiple years of data would be required in order to definitively state its population status, i.e. increasing, decreasing or stable. All study sites, specifically all natural sites (such as CNA, CNB, BK1A and BK1B), should be protected.

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Table 2.1. Locations of all permanently tagged Salix jejuna sites.

Site	Number of plants tagged in 2004	GPS coordinates	Disturbance
CNA (Cape Norman A)	40	21 U 0576165 5720124	Natural
CNB (Cape Norman B)	40	21 U 0575782 5718941	Natural
CNC (Cape Norman C)	40	21 U 0574770 5718917	Natural
CND (Cape Norman D)	40	21 U 0573683 5718508	Anthropogenic
BH (Boat Harbour)	40	21 U 0569311 5716001	Natural
BK1A (Big Brook 1A)	40	21 U 0554559 5706559	Natural
BK1B (Big Brook 1B)	40	21 U 0554390 5706424	Natural
BK39 (Big Brook 39)	40	21 U 0563024 5710086	Natural

Table 2.2. Brief outline of Salix jejuna reproductive events in 2004.

2004 summer	Description of plants
1st week of June	Males have developing catkins. Female catkins too small to see.
2nd week of June	Male catkins are fully developed and just beginning to release pollen. Female catkins starting to develop.
3rd week of June	Male catkins are fully developed and releasing pollen. Female catkins starting to develop.
4th week of June	NA
1st week of July	Male catkins are spent, all pollen has been released. Female catkins are fully developed and ovaries have started to swell but are not dehiscing yet.
2nd week of July	Male catkins are spent, all pollen has been released. Female catkins are fully developed and ovaries have started to swell but are not dehiscing yet.
3rd week of July	NA
4th week of July	NA
1st week of August	Female catkins are developed and ovaries are dehiscing. There was plenty of seed available for collection.
2nd week of August	NA
3rd week of August	Females are still continuing to release seed.
4th week of August	NA
1st-4th week of September	NA
1st week of October	Females are spent; all seed has been released. The leaves of all plants have yellowed and mostly fallen off.

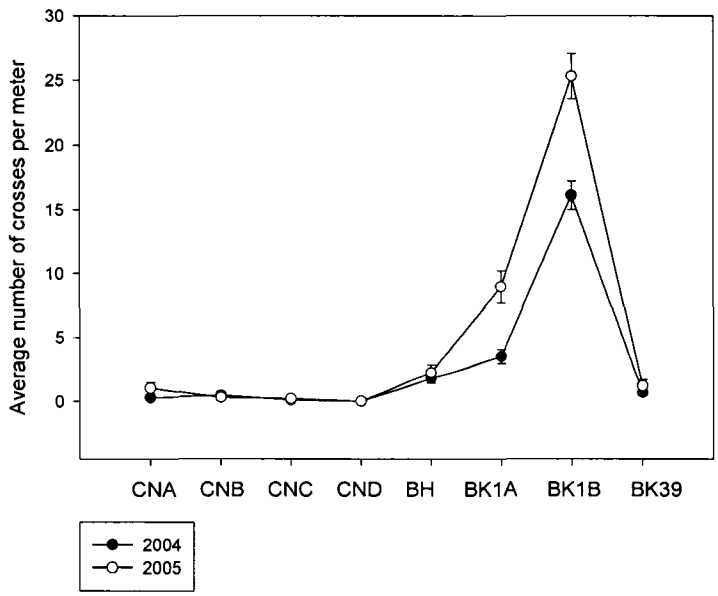

Figure 2.1: Site density (measured as mean number of stem or root crosses per meter of transect) of all currently established S. jejuna sites. Refer to Table 2.2 for site abbreviations. Error bars signify standard error.

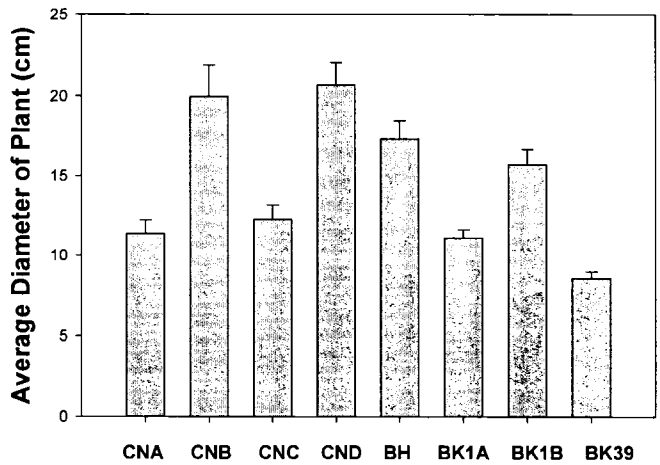

Figure 2.2: Average diameter of S. jejuna between all study sites (refer to Table 2.2 for site abbreviations. Error bars signify standard error.

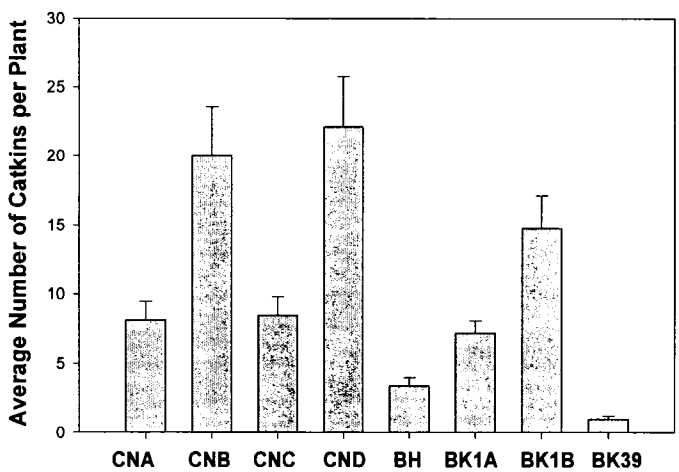

Figure 2.3: Average number of immature catkins per plant in June (2004 and 2005 data pooled). Refer to Table 2.2 for site abbreviations. Error bars signify standard error.

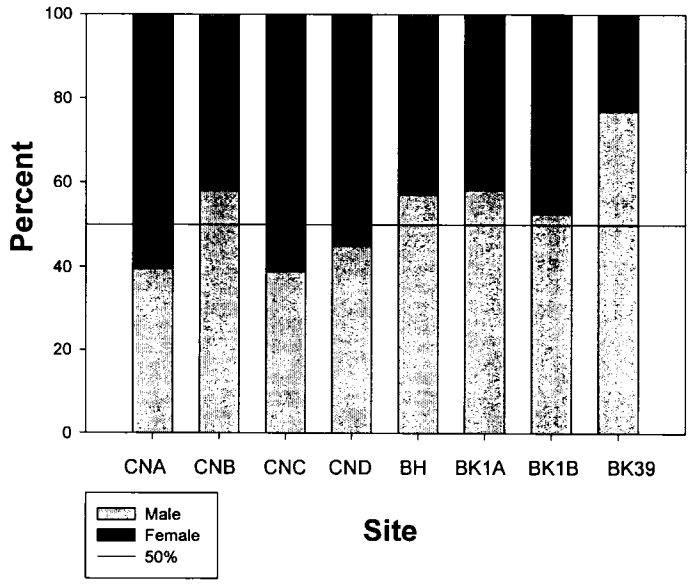

Figure 2.4: Percent of male and female Salix jejuna plants per site. Refer to Table 2.2 for site abbreviations.

Chapter 3

Ex situ methods for Endangered Limestone Barrens Plants

Abstract: Reliable *ex situ* protocols for the growth and maintenance of Braya longii (endangered), Braya fernaldii (threatened) (Brassicaceae) and Salix jejuna (endangered) (Salicaceae), endemic to the Limestone Barrens were developed. Based on nutrient and particle size analysis of natural soils within braya's natural distribution, a soil mix containing a 1:1 mix of braya mix to crushed limestone delivered desirable growth. In the 1:1 soil mix the average basal diameter of both braya species combined is 1.2 mm after one year and 2.0 mm after two years. The growth was greater than that for the natural braya seedlings, but not as large as the plants grown in braya mix, which had an average basal diameter of 2.4 mm in one year and 4.0 mm after two years. Cuttings of S. jejuna grew exceptionally well and had a 69% survival rate. Germination experiments on Salix jejuna seed revealed that it is indeed viable for up to 9 months if stored in a freezer at -20 ^{0}C. In light of this a long-term seed gene bank of S. jejuna seed may be possible.

Key words: Braya longii, Braya fernaldii, Salix jejuna, *ex situ* conservation, endangered plants, seed gene banking, seed longevity and limestone barrens.

Ex situ strategies for conservation

E*x situ* conservation is important in the management and protection of an endangered plant for several reasons: first, an *ex situ* population will be invaluable as a failsafe in the event that the natural populations are extirpated; second, it can supply the plant material needed for reintroduction; and third, it can also be used for experimentation to reduce impact on natural populations. Moreover, *ex situ* conservation is one of the 6 primary strategies listed in the National Recovery Plan for both Braya species (Appendix 1) (Hermanutz et. al., 2002). *Ex situ* populations should arrest the erosion of the genetic diversity that may be occurring in nature, giving species a long-term chance of survival (Given, 1994; Heywood, 1990).

Two major *ex situ* conservation strategies were used in this study: establishment/maintenance of live plants and seed gene banking. The ability to maintain an *ex situ* population through the persistence of live plants is tremendously important. This can be accomplished by germination of seeds in appropriate soil mixes, as can be used for herbaceous plants that produce viable seed. Persistence of live plants can also be accomplished by means of cuttings, as is generally done for many woody plants. Seed gene banking, a long-term storage of seeds in a freezer is also a valuable strategy to use in *ex situ* conservation but is only useful if protocols exist for successfully growing out the species. The potential to bank seed successfully plays a critical role in the ability to develop and maintain an *ex situ* population that preserves individuals from a species entire distribution.

Study Species

The Limestone Barrens of the Great Northern Peninsula of Newfoundland (Canada) contain three world endemics: Salix jejuna (endangered), Braya longii (endangered) and Braya fernaldii (threatened). Braya longii and B. fernaldii are small herbaceous perennials that die back to the crown every year. They are considered to be long-lived plants, most likely decades old, but the average life span is still unknown. As of 2005 eight years of long-term data has been collected; further monitoring is continuing in order to obtain this information. Not only do these plants have long lives, they also have an extensive seed bank (Hermanutz et. al., 2002). Since there is such an abundance of seed, the *ex situ* conservation strategy for braya concentrated on developing soil mixes and protocols that produced slow and sustained growth.

Salix jejuna (the Barren's willow) are small prostrate shrubs with a very different life history strategy than the braya species. Very little was known about S. jejuna at the beginning of this study (Djan-Chékar et al., 2003). The adult plants are long-lived and capable of asexual clonal growth, but little was known about the viability and longevity of the seed. The Barren's willow, if similar to most other arctic or arctic-like willows, was assumed to have low seed longevity and viability. However, this needed to be investigated. Consequently, the *ex situ* conservation strategy for S. jejuna concentrated on developing protocols for vegetative propagation and investigating seed longevity and viability. The opportunity to compare and contrast such different plants growing in similar habitat was a major reason for including both Braya and Salix in this study.

Development of an optimal soil mix for the persistence of live plants

Soil Geology and Chemistry

The bedrock geology of the Limestone Barrens of the Great Northern Peninsula is composed of carbonate shelf rock, mainly limestone dolomite and interbeds of shale (Greene, 2002). The Quaternary geology of the area comprises glacial diamicton (till), marine sediments and weathered bedrock, which has very little vegetation coverage. Preliminary substrate analysis was completed by Greene (2002); soil nutrients and texture were analyzed from a limited number of braya sites. He concluded that substrate characteristics did affect the population structure of braya. The analysis also focused on where braya were most likely to occupy among the three dominant substrate types: frost sorted circles (whether it is the border zone, intermediate zone or central zone), unsorted diamicton and anthropogenic substrate. Only 4 sites were used in this study: SCN, SCD, ANC and YPD (Greene, 2002; Noel, 2000; Hermanutz and Parsons, 2002). The majority of the samples were mainly composed of silt and clay, however, an accurate comparison of the soil between species and disturbed vs natural sites was unclear. A more comprehensive analysis from a greater number of sites is required in order to determine an optimal soil mix for *ex situ* persistence of both species.

The effect of nutrient availability is not the same for all plant species; however, an increase in nutrient availability will generally produce larger more robust plants. This relationship is important to understand since an optimal soil mix for both braya species is

not one that will produce large plants, but on the contrary, will produce small, more natural sized plants. A study was done on sub-Arctic heath, which looked at how plants would react to a combination of nutrient addition as well as warming (warming was done to emulate why there may be an acceleration of decomposition and therefore nutrient availability) (Richardson et. al., 2002). In this study an inorganic fertilizer solution was used which increased the amount of nitrogen, phosphorus and potassium. An increase in nutrient availability was found to affect the aboveground biomass of both dominant (dwarf shrubs) and subordinate (grasses and mosses) plants, the latter being of greater magnitude (Richardson et. al., 2002). Therefore, lowering the nutrient availability in plants may produce the opposite effect: smaller plants.

Seed Gene Banking

It is generally recognized that most willow seed shows high viability immediately after dispersal (Young and Clements, 2003; Maroder et al., 2000 and Douglas, 1995), but is only viable for a short time period (Arya et al., 1988 and Maroder et al., 2000). In fact, many germination studies on willows are conducted with seed harvested and used immediately for that reason (Young and Clements, 2003). Furthermore, willow seeds entirely lose their viability within a month at room temperature (Arya et al., 1988; Maroder et al., 2000). Even at -20^0C, the temperature typically used for seed gene banking, less than 50% of seeds for some species remain viable after just a few months in storage (Wood, et al., 2003 and Maroder et al., 2000).

There is also debate as to whether willow seed can survive desiccation; some studies have shown that lowering the moisture content in a seed will actually prolong its viability when done in combination with freezing (Maroder et al., 2000), while other studies suggest that any desiccation at all will reduce the viability of seeds tested immediately but increase the viability of seeds tested over time: in other words desiccation will increase the longevity (Wood et al., 2003). For this reason any preliminary studies on the viability and longevity of willow seed should include a combination of drying and freezing.

Objectives

The research objective in this study includes developing protocols for the persistence of live plants and determining if seed gene banking is possible for all three species. First, soil mixes were developed, which facilitate a more natural, slow, and sustained growth for both braya species. These soil mixes were based on chemical and textural analysis of the natural substrate. Second, the viability and longevity of Salix jejuna seed was evaluated. The optimal storage conditions for sustained longevity of S. jejuna seed, i.e. drying, freezing or a combination of both, were tested for the future purpose of seed gene banking.

Materials and Methods

Persistence of Live Plants

Collection of soil samples

In order to develop optimal soil mixes for the braya species soil cores were taken throughout the study area (Appendix 3) and analyzed for nutrient content, organic matter, soil moisture and grain size (textural analysis) to understand the substrate affinities and variability for use in establishing viable *ex situ* populations of both Braya longii and Braya fernaldii. Soil cores were also analyzed for pH, % nitrogen, calcium (ppm), phosphorus (ppm), potassium (ppm), magnesium (ppm), organic matter, particle size and moisture content (Appendices 3 and 4). Not all samples could be analyzed for nutrients due to the expense.

Developed Soil Mixes

Based on the results of soil analysis, several soil mixes were developed in an attempt to closely emulate the natural soil. The two variables that were felt to be most important were particle size and moisture retention (or drainage). As shown below the particle size from field sites differed dramatically from the artificial soil mix used in the past for *ex situ* braya studies. Particle size was also a variable that could be controlled in order to determine the effects it has on plant growth.

Three soil mixes were made with varying combinations of braya mix, an alpine-like soil mix composed of a 10:1 mix of topsoil to leaf mulch (Appendix 2) and

crushed/powdered limestone. The limestone was crushed using a Massco 4X6 crusher (also known as a jaw crusher) and a Bico pulverizer until it was all within the silt/clay particle size category. These three soil mixes are: pure braya mix, 1:1 braya mix to crushed limestone and 3:1 braya mix to crushed limestone (Appendix 2). By experimenting with the particle size of soil mixes drainage was clearly affected.

Previous attempts to germinate and grow braya in soil

The extremely rapid growth shown by both braya species during the summer of 2003 was severely checked by an infestation of thrips (Heliothrips sp. or Hercinothrips sp.). This insect is a serious and very common pest of the North American flower and nursery industry but is normally of little concern in Newfoundland. Recent warm summers seem to have brought about an upsurge (Dixon, 2003, pers. comm.) but cooler late season conditions appeared to have eradicated them.

In November 2003, approximately 130 braya (including representatives from both species) were moved into the greenhouse in order to determine if inside over-wintering would be successful. This triggered a hatch of aphid eggs that had been laid during the summer. The infestation multiplied rapidly and was manually controlled pending the build-up of an introduced bio-control organism (Aphidoletes aphidimyza). When it became evident that Aphidoletes were not successful in controlling the Aphids (due to the sheer number of predators that would have been required to get them under control) another bio-control organism (lady buds) were brought into the greenhouse. While

successful at controlling the Aphid populations on some of the other plant species housed in the greenhouse, they were not successful in controlling the aphids on the Braya.

Therefore, at the end of January (2004) all of the infested braya were moved into the cold house, as adult aphids will die when exposed to winter temperatures. While the shock of sudden winter may have killed some plants as well it was obvious that if left inside these braya would eventually die due to the intense aphid pressure.

Since the aphid infestation in 2003 all braya, including those planted before the aphid problem, as well as all braya planted in 2004, have been kept outside in a sunken bed. While some aphids are still present on the adult braya plants the numbers are far less than when the plants were kept inside the greenhouse. It is unrealistic to expect to get rid of all aphids so the long-term plan for pest management is to allow an acceptable number of them. Also no thrips were noted on the braya in 2004, this might have only been a problem in 2003. From 2004 on all braya that have germinated have grown and over-wintered in either one of the outside sunken beds at the MUN Botanical Garden or in the coldhouse at the same facility. At the end of the growing season (both in 2004 and 2005) all braya planted since the summer of 2003 were measured and recorded for the following: basal diameter (cm), length of longest leaf (cm), stage (single or multiple flowering or non-flowering), # of flowering stalks, # of fruiting stalks, damage due to insects and presence of absence of aphids. These variables were compared to determine growth rate and reproductive capacity in the previously used alpine soil mix.

Germination of braya seed in developed soil mixes

Since braya are small plants and grow for a long period of time and have a long pre-reproductive period in their natural environment, the soil mixes were developed to slow down the rapid growth that had previously been seen at the Botanical Garden. The goal was to emulate more 'natural growth'. Two germination trials of braya in varying soil mixes were conducted in 2004. The first trial was conducted in May 2004 after there was no further risk of snow and frost. The second trial was conducted in July after preliminary observations on the first trial determined that germination was indeed successful in the soil mixes. The first trial included seed from 4 Braya longii populations: YPD, LCD, ShoCoD and APN, and 4 Braya fernaldii populations: BCD, PAC, CNN and WPD. Five seeds per site were used in each of the following soil mixtures: pure braya mix, 1:1 braya mix to crushed limestone and 3:1 braya mix to crushed limestone. In total 120 seeds were used in this trial.

The second trial only included 2 soil mixes: the pure braya mix and the 1:1 braya mix to crushed limestone mix. The braya mix was included in this second trial as a necessary control and the 1:1 mix was chosen because preliminary observations determined that the braya plants in this mix most closely emulated the desired growth. In total 160 seeds were used in this trial: 10 seeds per site in each of the two soil mixes. The same sites used in the first germination trial were also used in the second germination trial. This germination experiment (both trial 1 and trial 2) tested the % germination as well as overall growth of braya seedlings in different soil mixes.

Salix jejuna Cuttings

In order to establish a viable *ex situ* population of Salix jejuna vegetative propagation protocols needed to be devised. In the winter of 2003 representatives of S. jejuna, previously collected and growing at the MUN Botanical Garden, were used in a preliminary study. Three sets of cuttings from these plants were taken in the fall of 2003. In each set 20 cuttings (5-10 cm) were placed (without rooting hormone) into a 50/50 mix of perlite/promix in a '67' plug tray. Cuttings were placed in a 20^0C green house under mist for a month, after which they were taken out of the mist and moved into a raised bed outside at the Botanical Garden. At this stage they were watered with ¼ strength 20/20/20 fertilizer.

Based on the results of this early experiment, cuttings from wild S. jejuna plants were collected in June of 2004, and again in 2005. Twenty tagged individuals at each of the 8 currently marked sites in 2004 were selected and one 5-10 cm cutting was obtained per individual. All cuttings were cleaned in water to remove adhering soil. Any remaining catkins (both male and female) were removed so the cuttings would divert their resources towards growth and not reproduction. Cuttings were then placed into Pro Mix® potting soil in standard 67 plug, 3' flats. These were placed outside in a makeshift enclosure and misted twice daily until they could be taken to the Botanical Garden. The flats of cuttings were then placed outside in a sunken bed.

Seed Gene Banking

Collection of Salix jejuna Seed

In August of 2004 eight to ten S. jejuna catkins per site were collected from tagged individuals at established sites (see Chapter 2). Catkins were selected only if they had already begun to naturally dehisce, meaning 1 or more ovaries had begun to split and release its seed. Catkins were then placed in dry Petri dishes and allowed to naturally open and release seed. Seed was collected for four days; this time variable was known as the 'day of seed collection'. On any of the 4 days after harvesting, all released seed was collected and split into 1 of 6 germination trials.

Germination success was tested in 6 different trials:

1. Seeds immediately germinated on a Petri dish
2. Seeds frozen for a week then germinated on a Petri dish
3. Seeds frozen for a month then germinated on a Petri dish
4. Seeds frozen 9 months then germinated on a Petri dish
5. Seeds dried at room temperature for a week then germinated on a Petri dish
6. Seeds dried at room temperature for a week, frozen for 9 months and then germinated on a Petri dish

Germination conditions

As stated earlier nothing was known about the requirements for Salix jejuna germination and optimal seedling growth. With this in mind a small trial was done to determine if there would be a difference in the percent germination of Salix jejuna seeds if they were germinated on a Petri dish vs germinated in potting soil. Seed was collected

from 2 sites on the Northern Peninsula: Cape Norman site A (CNA) and Big Brook site 1 B (BK1B) (see Chapter 2 for site locations). In total 392 seeds were collected from 24 individuals in August of 2004.

Data Analysis

Minitab Statistical Software for Windows, Version 13.30 was used to carry out all statistical tests performed in this study. The General Linear Model (GLM) was used to carry out all statistical tests. For each test residuals were examined for normality, independence, and homogeneity to ensure that the assumptions assumed in statistical tests were not violated. If the data were found to be non-normal then it was randomized as randomization tests make no assumptions (Schneider, 2003). However, if the sample size and the F-value were large then randomization was not done, as the General Linear Model is known to be robust to violations of assumptions (Schneider, 2003).

Braya longii and B. fernaldii were compared whenever possible to determine if the variable 'species' affected any results. In these cases a nested GLM was used; site was always nested in species as none of the braya sites contain both species. P-values from these tests were used to determine whether differences between data were statistically significant when alpha was 5%.

Minitab was used to carry out general descriptive statistics. Sigma Plot for Windows, Version 3 was used to create all graphs and Microsoft Excel 2000 was used to organize all data.

Results

Soil Analysis

In order to develop optimal soil mixes samples were analyzed for moisture and particle size. Only a select few were analyzed for nutrients due to expense (Appendix 4). The particle size was significantly different between all braya sites; each particle size class (explanatory variable) was calculated separately with 'species' and 'site' being the descriptive variables (Table 3.1; Figure 3.1). A nested General Linear Model was used for this and all analysis; species and site were both used as descriptive variables. Since only either B. longii or B. fernaldii plants are found at any given site there was a lack of co-linearity in a non-nested design. In other words, half the sites contained one species and the other half contained another so the species could not be compared at the same time as site. In order to analyze both species together, to determine any species differences, site was nested within species. Particle size was significantly different among all sites; each particle size class (explanatory variable) was calculated separately with 'species' and 'site' being the descriptive variables (Table 3.1; Figure 3.1). Particle size class was quite variable between sites; the proportion of silt and clay was high at such natural sites as Airport Natural (79.50%) and Shoal Cove Natural (60.60%) and low at Airport Disturbed (12.99%) and Burnt Cape (9.66%), both disturbed sites. The residuals were normal and there were no significant interaction terms.

Soil moisture at braya sites was tested with 'site', 'species' and 'month' as descriptive variables. Due to a species*month interaction B. longii and B. fernaldii were

tested separately (i.e. the sites have different temporal patterns in soil moisture); in both cases the residuals were normal and there were no significant interactions. The site from which soil was collected significantly affected soil moisture for B. longii ($F_{[8,72]}$ =4.72 p<0.001) (Figure 3.2). Sandy Cove Disturbed (SCD) and Lion's Club Disturbed (LCD) were the wettest sites, with soil moistures of 46.02% and 39.71%, respectively. These sites are both disturbed and a large component of the substrate is peat, which may be why these sites can retain more moisture. The site with the lowest soil moisture was Yankee Point Disturbed (YPD), at 4.73%. These F and p values were recalculated for the variable 'site' because of the nested design. The soil moisture for B. longii was also significantly affected by month (nested in site) ($F_{[21,72]}$ =2.12 p=0.019). July was the driest month with only a 15% moisture level and October had the highest moisture content at 29%.

The site from which soil was collected significantly affected soil moisture for B. fernaldii as well ($F_{[4,57]}$ =0.99 p<0.001)(Figure 3.3). The F and p values were recalculated for the variable 'site' because of the nested design. Shoal Cove Natural (ShoCoN) was the wettest site, with a soil moisture level of 20.80% and Green Island Brook (GIB) was the driest at 11.08%. The soil moisture for B. fernaldii was also significantly affected by month (nested in site)($F_{[15,57]}$ =6.27 p<0.001). For B. fernaldii August was the driest month at 10.08%.

Soil moisture for Salix jejuna was significantly affected by site ($F_{[7,14]}$ =4.48 p=0.026) (Figure 3.4). Compared with braya sites soil moisture was much lower, ranging between 15-18%, with two exceptions. The lowest moisture level was at the disturbed

site (CND), at 7.06%, and it was also lower at the CNB site where most of the site is covered by large slabs of limestone. The residuals were normal and there were no significant interaction terms.

Braya growth in Alpine Mix

Size analyses, both differences in basal diameter and length of leaves, were conducted on braya that were sown in the summer of 2003. These plants were measured and compared in 2004 and 2005. Species and site were used as descriptive variables; the residuals were normal and there were no interaction terms. There was no difference between the basal diameters of the 1-year-old plants (4.5 mm, SE=0.17) vs the 2-year-old plants (5.1, SE=0.30), indicating that second year plants were not significantly larger than the first year plants. The plants appeared to quickly grow the first year and then did not add significant growth in their second year.

The basal diameter was significantly different between the two species ($F_{[1,290]}$ =4.19 p=0.0416). The F and p valued were recalculated due to the nested design; site was nested within species. The average basal diameter for B. longii was 4.4 mm (SE=0.17) and the average for B. fernaldii was 5.5 mm (SE=0.40). The basal diameter was also significantly different among the sites tested ($F_{[13,290]}$ =3.59 p<0.001) (Figure 3.5). The site with the largest basal diameter was Cape Norman (CNN)(9.0 mm); some of the sites with the smallest basal diameter included APD and LCD with a diameter of 3.4 mm and 3.6 mm, respectively.

The longest leaf was not significantly affected by either the species or site used. However, the 1-year-old plants had larger leaves than the 2-year-old plants ($F_{[1,321]}$ =23.13 $p<0.001$). The average value for longest leaf in 2004 was 9.2 mm (SE=0.25) and the average value for longest leaf for 2005 plants was 7.1 mm (SE=0.33). So not only did the basal diameter not change from year 1 to year 2, but the length of the leaves actually decreased.

Since it was important to understand the growth of braya in an *ex situ* environment, the stage of the plants, as well as size was analyzed. The four stages of braya (though they do not necessarily progress in a step-wise fashion) are single rosette non-flowering (SN), single rosette flowering (SF), multiple rosette non-flowering (MN) and multiple rosette flowering (MF) (Hermanutz et al., 2005). Stages rather than ages were used because these herbaceous plants die off to the crown yearly and cannot be aged. While the stages of the *ex situ* braya (SN, MN, SF and MF) in the alpine mix could not be tested statistically, because overall proportion provides only one value, it is obvious that many plants were either in the multiple rosette stage and/or flowering after 1 year (Figure 3.6). This means that the plants were advancing to a multiple stage and in many cases flowering after only a year of growth. Also the trend from SN in 2004 to SF in 2005 should be noted as this also indicates that the plants were quickly progressing from non-flowering plants to flowering plants. Therefore the plants did not continue to grow in size but changed from juvenile to mature (i.e. reproductive).

Braya Germination in developed soil mixes

In this experiment all braya planted in 2004 in one of the three developed soil mixes were used. These soil mixes are 1:1 mix of braya mix to crushed limestone. 3:1 mix of braya mix to crushed limestone and 100% braya mix. The descriptive variables used were 'soil mix', 'year', 'species' and 'site' (nested within species). The residuals were normal and no interaction terms were significant. The basal diameter was not significantly affected by site.

The basal diameter of braya was significantly affected by soil mix ($F_{[2,43]}$ =14.47 $p<0.001$) (Figure 3.7). The braya in the 1:1 soil mix had the smallest average basal diameter and the braya in the braya mix has the largest. The basal diameter was also significantly affected by year ($F_{[1,43]}$ =12.73 $p<0.01$), the basal diameter was larger in the second year of growth for all three soil mixes. The basal diameter was also significantly affected by the species; B. longii had an average BD of 3.1 mm (SE=0.42) and B. fernaldii had an average BD of 3.6 mm (SE=0.56).

The length of the longest leaf was analyzed with the following descriptive variables: 'soil mix', 'year', 'species' and 'site' (nested within species). The residuals were normal and no interaction terms were significant. Year, species and site did not affect the length of the braya leaves (all $p>0.05$). Unlike BD the length of the leaves were not affected by the year. However, the length of the longest leaf was significantly affected by the soil mix ($F_{[2,42]}$ =19.46 $p<0.001$) (Figure 3.7).

Salix jejuna cuttings

Cutting survival was very high among all the 2004 cuttings; when recorded in 2005 the overall survival rate was 69%. The site at which the cuttings were taken significantly affected their survival ($F_{[7,159]}$ =11.37 $p<0.001$) (Figure 3.8); CNB had the highest survival rate at 90%. These differences may be influenced more by the size of the cuttings (large cuttings of 10-20 cm could not be collected from sites with small plants) than by the actual site they were taken from. In hindsight cutting length should have been used as a covariate, however while the cutting length was noticeably different among sites the exact difference could not be calculated as it was not recorded. Since the survival is reported as overall percent the standard error (SE) could not be calculated.

Germination of Salix jejuna Seed

Percent germination of Salix jejuna seed was very high in all of the germination trials from 2004. The 'treatment' given to the seeds significantly affected the percent germination ($F_{[5,93]}$ =3.53 $p=0.006$) (Table 3.2). Seeds germinated immediately had the highest germination rate (81%) while seeds frozen for 9 months had the lowest germination rate, at 48%. It should be noted that 1 of the 8 sites, the only anthropogenically disturbed site, CND, could not be included in the germination trials. While the plants at the CND site had a large number of catkins early in the year they produced virtually no seed.

Due to a different number of replicates per site (due to availability of seed) the site variable was analyzed separately for all treatments involved. The percent

germination was not significantly affected by site in any of the 6 treatments. Residuals were normal with no significant interactions.

Conditions for germination

A small germination trial was done to determine if there would be a difference in the percent germination of Salix jejuna seeds if they were germinated in Petri dishes vs germinated in potting soil. There was no difference between these two trials; seeds germinated equally on the Petri dishes as they did in the soil (40% and 38%, respectively). The site at which the seed was collected was not significant either.

Discussion

Persistence of Live Plants

Braya longii and Braya fernaldii

All braya sown in standard alpine mix in 2002 died; therefore there was a need to develop an appropriate soil mix to support *ex situ* populations of braya. Previous attempts to grow braya in an alpine mix produced plants with an average basal diameter (1 year old and 2 year old plants combined) of 4.4 mm for B. longii and 5.5 mm for B. fernaldii. While these sizes may seem small compared to other plants it is actually quite large for naturally growing braya.

In comparison, Tilley (2003) established seeded plots of both B. longii and B. fernaldii *in situ* in 2002. The braya in these plots have been measured for basal diameter (BD) and longest leaf from 2003 to 2005. As of 2005 the three-year-old plants have an average BD of 1.0 mm for B. longii and 1.1 mm for B. fernaldii. In essence the 1 and 2-year-old *ex situ* braya grown in alpine mix had basal diameters that were 4 to 5 times larger than the 3-year-old plants grown *in situ*.

Also the braya plants grown in the alpine mix were either flowering and/or developed multiple rosettes after only one year of growth. This is strikingly different from the 3-year-old braya seeded *in situ*, which were all single non-flowering plants (Tilley, 2005; unpublished data).

Selecting for rapid growth or other select traits is widely done in the horticultural industry in order to produce desired plants. However, selecting for rapid growth in an *ex situ* population can be disadvantageous as that will drastically alter the genetic diversity

in the population. Ultimately the goal of *ex situ* conservation is to establish off site populations that as close as possible emulate the natural populations. Therefore all future braya grown for the maintenance of the *ex situ* population should be grown in the 1:1 soil mix.

Salix jejuna

For Salix jejuna the focus on developing populations of live plants (establishing an *ex situ* population) was on developing a protocol for the survival of cuttings. Cuttings from naturally growing Salix jejuna grew best in an alpine-like soil mix (braya mix, Appendix 2). These cuttings had an overall 69% survival rate. This survival rate is not uncommon for willow species; it has been stated to be as high as 85-99% (Maschinski et al., 2004). The ability to successfully establish S. jejuna from cuttings is extremely important for the establishment of a viable *ex situ* population (Hagen, 2002). Harvesting cuttings from the *in situ* plants allows for 1) the parent plant to remain on site and 2) an identical copy of all plants harvested in the off site population.

Seed gene banking

While it had already been determined that both Braya longii and B. fernaldii seeds remain viable for several years if stored in a freezer at -20^{0}C (Hermanutz and Parsons, 2003), little was known about S. jejuna seed.

The viability and longevity of S. jejuna seed was investigated in order to determine if seed gene banking would be possible with this species. Originally Salix

jejuna seed was assumed to have low longevity and viability like many willows (Arya et al., 1988 and Maroder et al., 2000); however the results of the germination study provided evidence that the seed is indeed viable for up to 9 months if stored in a freezer at -20^{0}C. While the immediate germination success of many artic/alpine willow species is known to be high most have poor seed longevity, in most cases seed must be germinated immediately or it will die (Young and Clements, 2003; Maroder et al., 2000 and Arya et al. 1988), however this was not the case for S. jejuna.

As expected, the germination success of S. jejuna seed was very high initially; in fact it was 81% when all sites were combined. Other studies have shown similar results of 96% to 100% germination success of seed tested immediately (Maroder et al., 2000 and Arya et al. 1988). And while the high germination success of S. jejuna was not unexpected the maintained viability of the seed was. Even the seed that was frozen for 9 months still showed a 48% germination rate, exceptionally high for a willow. In fact other Salix species seed shows a lower than 50% germination rate after only a few months in frozen storage (Wood, et al., 2003 and Maroder et al., 2000). These results suggest that a seed bank of S. jejuna may be possible, this would greatly affect the *ex situ* collection. Furthermore, if a transient (1 year) or persistent (longer than 1 year) seed bank is present on site then all suitable habitat in close proximity to adult plants may be designated as critical habitat since any seeds contained in the soil could germinate and develop into adult plants. A more extensive and long-term trial would be required in order to determine if long-term seed gene banking is possible for S. jejuna. For this

purpose S. jejuna seed was collected from all available sites in 2005 and stored in a freezer for future studies.

Summary

Mimicking the natural soil for the *ex situ* populations delivered desirable growth and development. The braya seeds sown in this mix grew into healthy plants that closely emulated the natural growth rate found in the *in situ* populations. The artificial soils appear to be an appropriate surrogate for Limestone Barrens species, and may be appropriate for many other calciphiles. Developing protocols for the establishment of S. jejuna populations (by means of cuttings) was equally successful. Cuttings grew exceptionally well and had a high (69%) survival rate after 1 year.

It had already been determined that seed gene banking was possible for both Braya longii and B. fernaldii (Hermanutz and Parsons, 2003), however it was originally assumed that Salix jejuna seed would have low longevity and viability as with many willows (Arya et al., 1988 and Maroder et al., 2000). The results of the germination study provided evidence that the seed is indeed viable for up to 9 months if stored in a freezer at -20^{0}C. In light of this a seed gene bank may be possible with S. jejuna seed. However, 48 % viability after 9 months suggests that seed would need to be replenished yearly or a more advanced method, such as cryopreservation, may be required in order to increase longevity.

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Table 3.1: Particle size analysis for braya soil. F and P-values from a general linear model. * indicates F values recalculated because of nested design.

Particle Size Class	Descriptive Variable	Residuals	F	P
Silt/Clay (<0.0625 mm)	Species	Normal	$F_{1,77}$= 41.47*	P<0.001*
	Site (Species)		$F_{11,77}$= 45.60	P<0.001
Fine sand (0.0625-0.25 mm)	Species	Normal	$F_{1,77}$=1.732*	P=0.1921*
	Site (Species)		$F_{11,77}$=8.97	P<0.001
Medium sand (0.25-0.5 mm)	Species	Normal	$F_{1,77}$=0.83*	P=0.3651*
	Site (Species)		$F_{11,77}$=5.46	P<0.001
Coarse sand (0.5-2 mm)	Species	Normal	$F_{1,77}$=6.177*	P=0.0151*
	Site (Species)		$F_{11,77}$=17.84	P<0.001
Gravel (>2 mm)	Species	Normal	$F_{1,77}$=1.139*	P=0.2892*
	Site (Species)		$F_{11,77}$=3.61	P<0.001

Table 3.2: Germination success of Salix jejuna under various treatments.

SE = standard error.

Treatment	Percent germination	SE
Seed germinated immediately	81%	5
Seed dried for 1 week	80%	6
Seed frozen for a week	75%	8
Seed frozen for a month	75%	8
Seed frozen for 9 months	48%	8
Seed dried for 1 week and then frozen for 9 months	55%	9

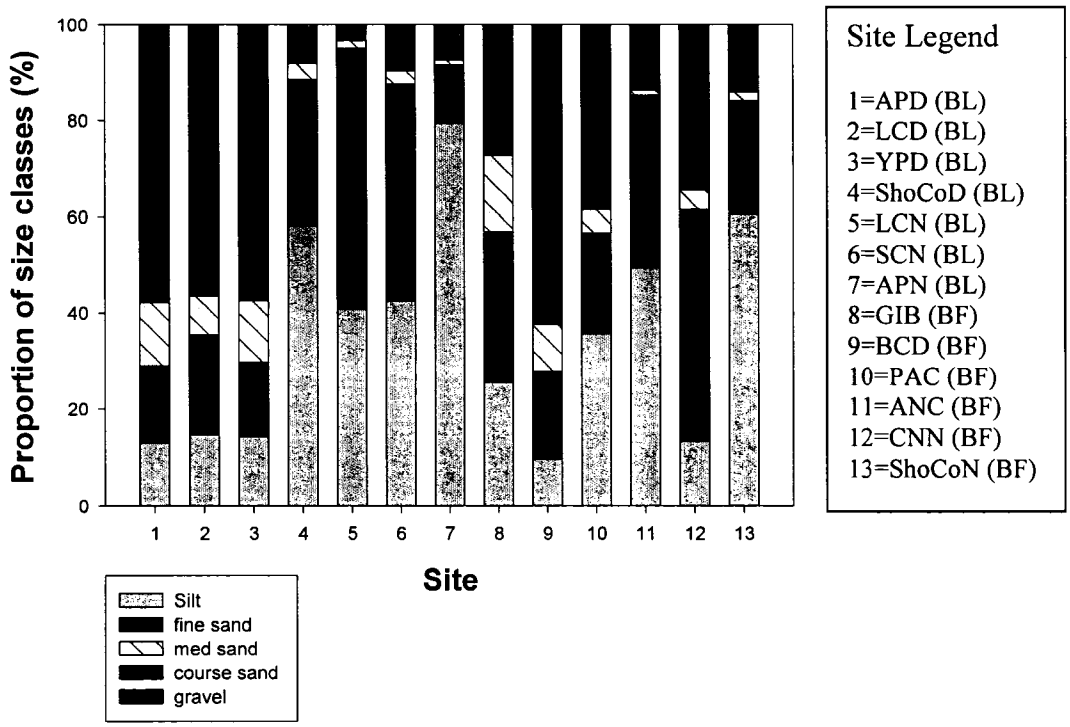

Figure 3.1: Particle size distribution of braya sites. Silt is < 0.0625 mm, fine sand is 0.0625-0.25 mm, medium sand is 0.25-0.5 mm, course sand is 0.5-2 mm and gravel is > 2 mm.

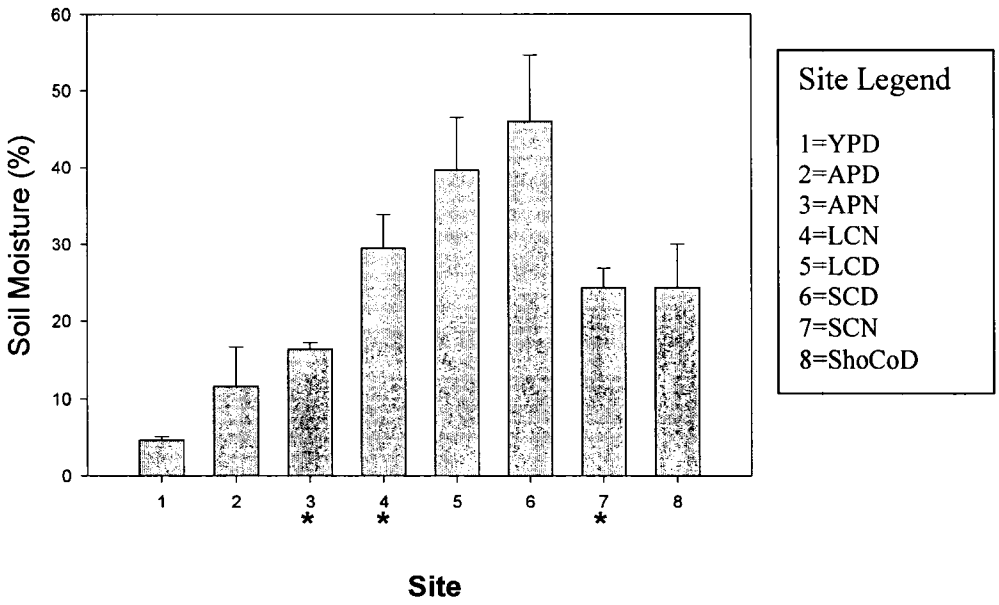

Figure 3.2: Soil Moisture of Braya longii sites, left to right indicates a South to North distribution. All sites marked with an asterix (*) are natural sites. Refer to Table 1.1 for site abbreviations. Error bars signify standard error.

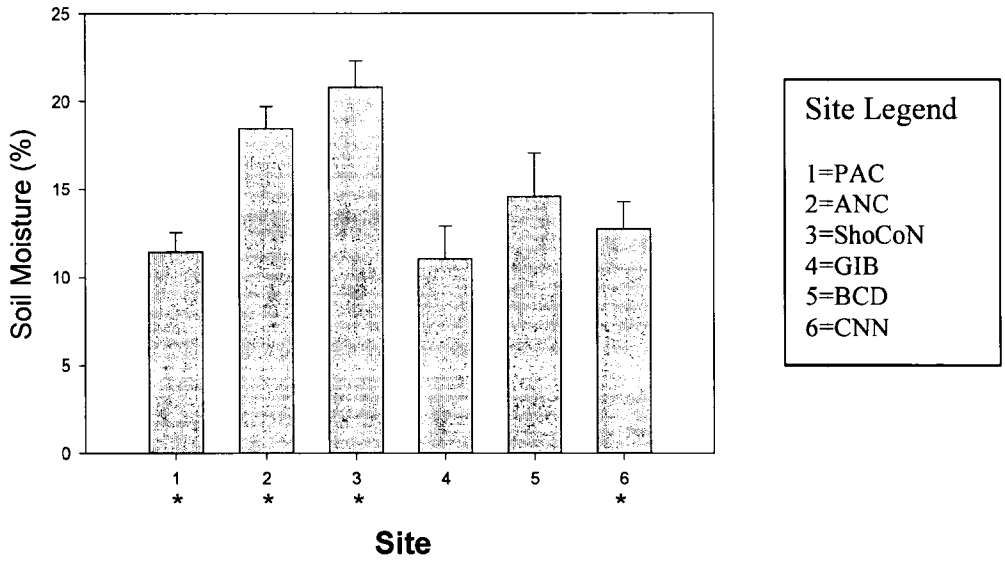

Figure 3.3: Percent soil moisture of adjacent Braya fernaldii sites, left to right indicates a South to North distribution. All sites marked with an asterix (*) are natural sites. Refer to Table 1.1 for site abbreviations. Error bars signify standard error.

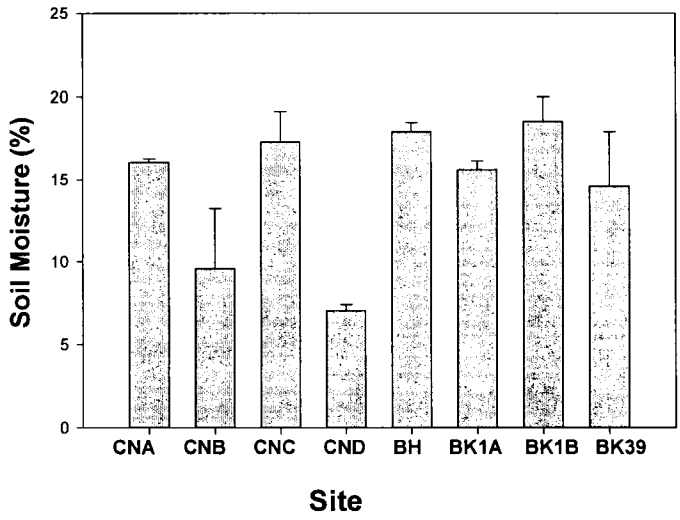

Figure 3.4: Percent soil moisture at all S. jejuna study sites. Error bars signify standard error.

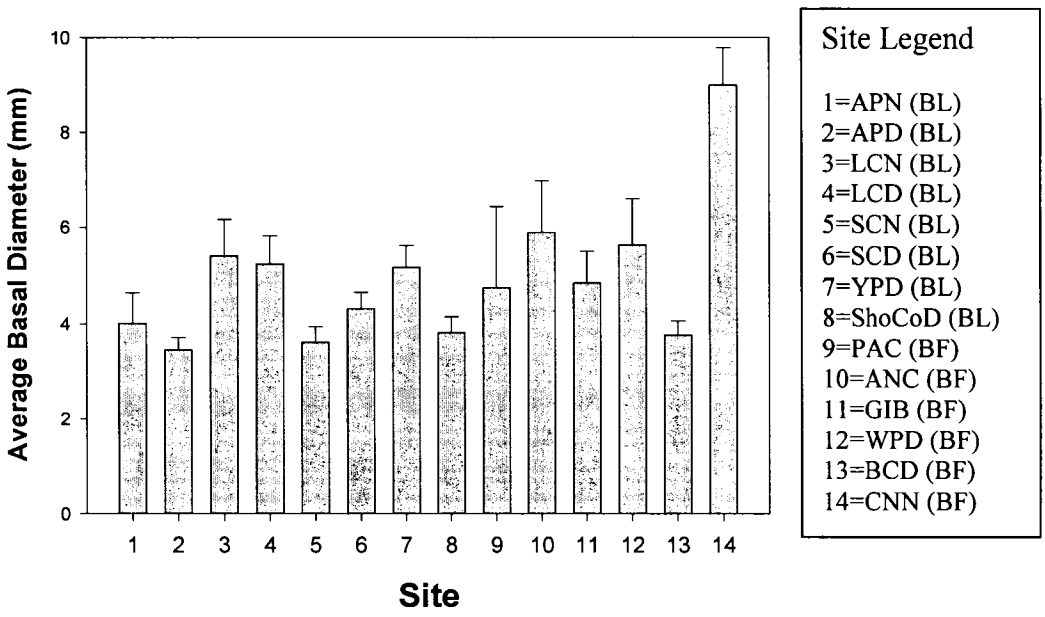

Figure 3.5: Mean basal diameter of braya per site (only 2003 braya planted in alpine mix). Error bars signify standard error.

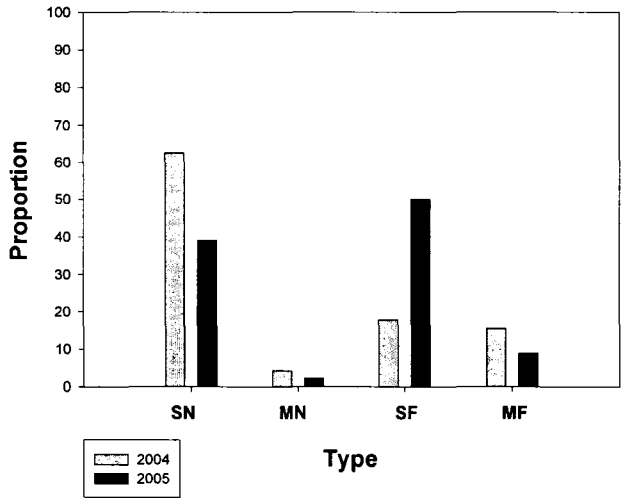

Figure 3.6: Proportion of stages for all *ex situ* braya planted in 2003 in alpine mix after 1 and 2 years of growth. SN= single rosette non-flowering, MN= multiple rosette non-flowering, SF= single rosette flowering and MF= multiple rosette flowering.

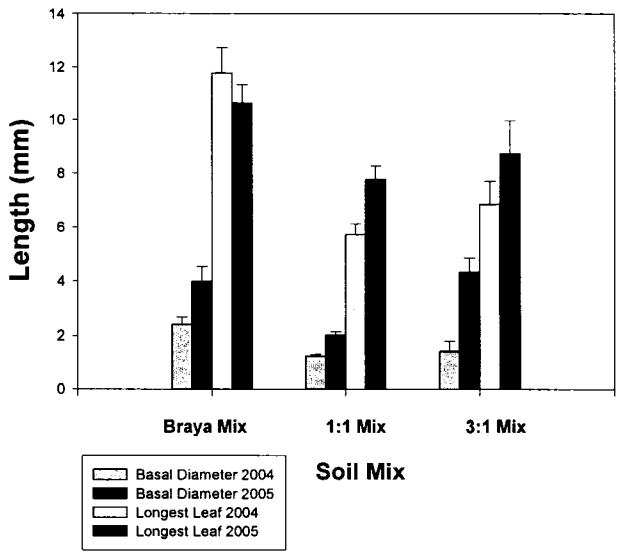

Figure 3.7: Size of 1-year-old braya planted in the summer of 2004 in developed soil mixes. Error bars signify standard error.

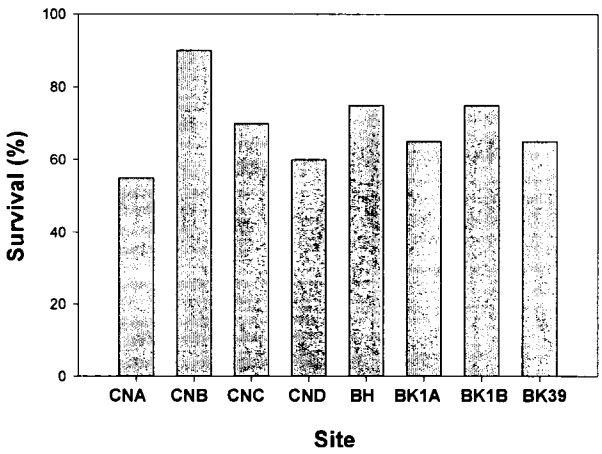

Figure 3.8: Overall percent survival of *ex situ* S. jejuna cuttings from 2004-2005. Error bars not obtainable as these represent site means.

Chapter 4

The use of Tissue Culture as a Tool for Conserving Endangered Plant Species

Abstract: Preliminary tissue culture protocols (with a focus on acclimatization) were developed for Braya longii (endangered), Braya fernaldii (threatened) and Salix jejuna (endangered); endemic species from the Great Northern Peninsula, Newfoundland (Canada). Initiation of braya was successful; however, preliminary protocols for successful acclimatization of either braya species were not established, highlighting the need for further experimentation into media requirements. Initiation and multiplication of S. jejuna was very successful in woody plant media (WPM); survival rate was as high as 94.8% three months after acclimatization from test tube to soil. The highest survival rate was in a 1:1 soil mix of an alpine soil mix to crushed limestone.

Key Words: Braya longii, Braya fernaldii, Salix jejuna, tissue culture, *ex situ* conservation, WPM, RLIBA and acclimatization.

Introduction

Tissue culture (or micropropagation) is an ideal technique to use when working with endangered plant species because a large number of plants can be produced from a small amount of parent material. Parent material (explants) may be from shoot tips, nodal cuttings, roots or seeds (Kyte and Kleyn, 1996). In many cases explants can be taken without killing the adult plant, thereby avoiding the further endangerment of the species. Some other advantages include: (i) plants can be maintained in culture indefinitely, because it is a sterile condition, free of pests and disease, (ii) it can be done at any time throughout the year, (iii) multiplication of plants in tissue culture is rapid (Chawla, 2002).

While the advantages of tissue culture are many, there are a few disadvantages. First, tissue culture is expensive and requires trained personnel and proper facilities. Second, there is a chance of undesired genetic variability, which is more common when using adventitious shoots or callus (Chawla, 2002). Third, when working with plants in tissue culture a major disadvantage is vitrification. Vitrification can be caused by repeated cycles of in vitro shoot multiplication; this gives plants water-soaked, slightly translucent leaves and will commonly lead to a decline in the rate of growth and eventually cause death. Last, while in theory tissue culture is a sterile environment free of pests and diseases there is always a chance for very high loss due to contamination.

Despite the known benefits the use of tissue culture in an *ex situ* conservation program is still comparatively rare. Most commonly it is used with endangered medicinal plants (such as the medicinal herb Spilanthes mauritiana) (Bais et al., 2002), plants that are valued in horticulture (such as the endangered orchid Ipsea malabarica)

(Martin and Madassery, 2005) or other industries (such as the Chinese aloe, Aloe vera L. var. chinensis) (Liao et al., 2004).

No previous tissue culture work has been done on Braya longii (endangered), Braya fernaldii (threatened) or Salix jejuna (endangered). Therefore, preliminary protocols for all stages of tissue culture needed to be developed. These included preparation of stock plants (stage 0), initiation (stage 1), mass proliferation (stage 2), rooting (stage 3) and acclimatization (stage 4) (Kyte and Kleyn, 1996).

Stages of Tissue culture

Preparation of plant material (stage 0) consists of sterilizing seeds or other explants, such as nodal cuttings (Kyte and Kleyn, 1996; Pruski, 2004). Once sterilized, these explants can then be inoculating onto the chosen media for a period of about 3 weeks (initiation or stage 1). A typical media will consist of macronutrients, micronutrients, a gelling agent and any growth regulators required by the specific plant. The multiplication stage (stage 2) can be done in several ways. If the plant grows by means of nodal branching then simply cutting the plants into nodal segments accomplishes the needed multiplication because each node would produce a new plant. If, on the other hand the plant grows by division of rosettes, then the axillary bud method could be used. When multiplying plants using this method it is essential to use the plant hormones cytokinin and auxin, usually in a 10:1 ratio. Rooting (stage 3) may be easy to establish in some plants and difficult in others. Nodal explants usually root well without any additional hormones; however explants produced from axillary bud multiplication

usually require cytokinin and auxin (Kyte and Kleyn, 1996). Adjustments of hormones may often be required for successful rooting. The final step in tissue culture is acclimatization (stage 4); here the plants are transferred from test tube to soil.

Transplanting delicate plants from an *in vitro* condition to soil can result in high levels of mortality if they are not allowed to gradually adjust from a high humidity environment (inside a test tube) to a less humid environment; because of this a common reason for mass death of transplants would be an improper protocol (i.e. not enough time for acclimatization or not enough humidity). Another reason for mortality during acclimatization is related to the health of the plants before transplanting; if the plants are not healthy they will most likely not survive this difficult step.

The ability to successfully grow and multiply plants using tissue culture methodology can be extremely beneficial when establishing a viable *ex situ* population of an endangered plant. Furthermore it is vital, especially if a conservation plan involves reintroduction of plants into native habitat, that the plants can be successfully acclimatized and transplanted into soil. Many tissue culture studies, specifically on endangered plants (Liao et al., 2004; Iriondo and Pérez, 1990), have highlighted acclimatization as the critical step in the tissue culture process.

As the acclimatization step is so crucial, the major focus of this work with B. longii, B. fernaldii, and S. jejuna, was on the acclimatization of these plants into soil. The ultimate goal of this work is to develop the methodology required to transplant these species into the natural environment.

Acclimatization within the Brassicaceae

No tissue culture studies have currently been done on the propagation of rare or endangered braya species; however, some have looked at the propagation of rare or endangered members of the Brassicaceae (Prevalek-Kozlina et al., 1997; Iriondo and Pérez, 1990). In all cases the survival of transplants is determined after the plants have fully adapted to the humidity and temperature of typical greenhouse conditions. In a study on Fibigia triquetra (a rare species from Southern Dalmatia, Europe, propagated for ornamental purposes) a 90% survival rate was obtained (Prevalek-Kozlina et al., 1997). However, in another study on Coronopus navasii, an endangered Brassicaceae species from Spain, the survival rate was much lower at 47% (Iriondo and Pérez, 1990). These studies both stated that the presence of a well-developed taproot was critical for survival.

Acclimatization within the Salicaceae

Previous tissue culture studies have been done on the propagation of rare or endangered willow species (Amo-Marco and Lledo, 1996; Neuner and Beiderbeck, 1993). Many of these studies have included acclimatization and survival rates. In a study on Salix tarraconensis (a threatened plant endemic to Spain) plant survival was 90% 4 weeks after transplanting (Amo-Marco and Lledo, 1996). In another study on Salix caprea (a pioneer shrub that cannot be propagated successfully by cuttings) the survival rate after 8 weeks of transplanting was between 32% and 94%. The difference in this study is attributed to the light regimes; the plants exposed to a 'long day' (at least 14 hours of light daily) had a much higher survival rate (Neuner and Beiderbeck, 1993). In a

study on 5 willow clones (Salix sp.), Bergman von Arnold and Eriksson (1985) found that genotype and shoot size played an important factor in survival.

Predictions

Salix jejuna are particularly suited to tissue culture based on the abundance of vegetative propagation that naturally occurs in the wild population. Because of this the need for an extensive natural seed bank may not be required as it can rely on vegetative propagation.

The exact opposite may be true for both Braya species. Neither of these plants reproduces vegetatively in the wild; however they both have extensive seed banks. Because of this they may rely on sexual reproduction as opposed to vegetative propagation.

Objectives

The use of tissue culture as a viable technique to develop reliable *ex situ* populations of all three listed species was assessed. Particular importance was placed on developing protocols for the successful acclimatization of both species from tissue culture to soil, as the future benefit from this work is the possibility of reintroduction.

Materials and Methods

Preparation of media for braya seeds

McCown's Woody Plant Basal Salt Mixture (WPM) was prepared for B. longii and B. fernaldii seeds (Appendix 5). This is a frequently used and well-known media (Lloyd and McCown, 1980). A modified media for the braya seeds did not include sucrose, because it is not required for seed germination (Pruski, 2004). 100 ml of media was adjusted to pH 5.7 and autoclaved in a 250 ml flask in preparation for making plates. When the media was cooled slightly 3-4 drops were allocated into each well of a 96 well germination plate using sterile pipettes in a laminar flow hood. The lid was placed on the well plates and allowed to cool for approximately 1 hour in the hood. The plates were then sealed with Parafilm®, labeled and placed in a coldroom at 5°C (Pruski, 2004).

Sterilization and inoculation of braya seeds

Seeds from two braya sites for each of the braya species were selected for tissue culture trials; one natural site and one anthropogenically disturbed site, for each species. These seeds were collected in 2004 and had been stored in a -20°C freezer until they were needed for experimentation. For B. longii the two sites selected were YPD (Yankee Point Disturbed) and APN (Airport Natural). For B. fernaldii, BCD (Burnt Cape Disturbed) and ANC (Anchor Point Natural) were selected. These sites were selected as they are the largest populations and therefore seed is not limiting, nor will seed harvest further threaten persistence (Hermanutz et al., 2005). Natural and disturbed sites were

chosen for each species in order to compare their success in tissue culture. Plants from disturbed sites are larger than plants from natural sites; this may affect either germination rate or growth rate in tissue culture. Also, the disturbed site BCD contains an unknown bacterial or viral pathogen that may negatively affect the germination success of seeds in tissue culture.

Thirty seeds from the 2004 *in situ* collection were used per site, 10 seeds from each of 3 different maternal lines. Two separate sterilization treatments were used; therefore the 10 seeds per maternal line were further split into two groups of 5 seeds, resulting in two equal sets of seeds. All seeds were scarified for approximately 10 seconds by rubbing them on fine sand paper. Groups of seeds were placed into fine mesh tea bags (5 seeds per bag), labeled with pencil and stapled shut.

All bags were rinsed in a mild 1% soap solution for 1 minute. Tepid water was then run over the sealed bags for 1 hour to remove soap residue and to allow the seeds to begin to imbibe so that sterilization would be effective. The bags were then placed in containers with a 1% bleach solution and placed on an agitator. A few drops of soap (1% solution) were added to each container to act as a surfactant. One set of seeds was soaked in the bleach solution for 10 minutes and the other was soaked for 15 minutes. After the specified amount of time on the agitator had elapsed the containers were placed in a flow hood and the bags were rinsed four times with ddH_2O to remove any remaining bleach.

In the flow hood, each bag was cut open and each of the 5 seeds was placed in an individual media filled well within the well plate. All tools were sterilized between bags. Once all the seeds were in the wells the plate was sealed with Parafilm® and placed in

the growth room. Plants were grown in a 16 hour day/8 hour night cycle at 23 ^{0}C and 28 ^{0}C, respectively.

Following germination, both braya species were grown in RLIBA rooting media (Appendix 5) (Collins and Phillips, 1982). This media was used because it contains the hormones auxin (IBA), and a cytokinin (3-aminopyridine), which promotes rooting. After all constituents were added to the media it was adjusted to pH 5.7 and 10 ml was allocated per test tube. The test tubes were allowed to cool before they were inoculated with explants (Pruski, 2004). Test tubes with RLIBA rooting media were used during the multiplication stage of braya.

Multiplication and Acclimatization of Braya

The leaves and root tips of the braya seedlings, once they were established in RLIBA media, were trimmed to stimulate growth and transferred to fresh media every 2-4 weeks. Since the growth form of braya is a rosette the axillary bud method was used in the multiplication stage. Either they were multiplied by separating axillary buds from each other or by using new plantlets that initiated from the roots.

Once sufficient numbers of braya had been multiplied, they were used in an acclimatization trial. The plants with the most developed root system and healthy leaves were chosen for this trial. In total, 250 plantlets were planted into soil; a third into a 1:1 mixture of powdered limestone to braya mix, a third into a 3:1 mixture of powdered limestone to braya mix and the last third into 100% braya mix (Appendix 2). These soils had been developed for braya and S. jejuna growth experiments and are based on their

natural substrate (see Chapter 3). The plants were then placed under mist at 25-30°C for the entire length of the experiment.

Sterilization of Salix explants

In December of 2004 stem cuttings of S. jejuna were taken from 3-year-old dormant adult plants growing in a sunken bed at the Memorial University Botanical Garden, St. John's. These plants were originally grown from cuttings taken from wild plants at Cape Norman site A (CNA) on the Great Northern Peninsula of Newfoundland. The cuttings were placed in water for 2 weeks at approximately 20 °C in order to allow the buds to flush and start to grow new leaves. Nodal cuttings were taken from the new shoots and placed in containers for sterilization.

These explants were rinsed for 1 minute in a mild 1% soap solution and then rinsed with water. Explants were then treated for 5 minutes with 2% household bleach (sodium hypochlorite) solution containing a wetting agent (a few drops of the 1% soap solution). After rinsing 3 times in sterile distilled water 2-3 explants were placed in standard test tubes, containing 10 ml of WPM. This media is used for other Salix species with much success (Amo-Marco and Lledo, 1996; Neuner and Beiderbeck, 1993).

Test tubes were then sealed with Parafilm® or medical tape and placed under light in a growth room. Plants were grown in a standard 16 hour day/8 hour night cycle at 23°C and 28°C, respectively.

Multiplication and Acclimatization of Salix jejuna

Every 2-4 weeks the plantlets were transferred to fresh media. When the plantlets were at least 3 cm long with 3-5 nodes the entire stem was cut up and all nodes were used as new explants in order to increase the number of plantlets. When approximately 500 plantlets were established in media their root tips and shoot tips were trimmed once more to encourage lateral growth, transferred to fresh media and were given 4 weeks to grow before subjected to the acclimatization trial.

In total, 462 plantlets were planted into soil; a third into a 1:1 mixture of powdered limestone to braya mix, a third into a 3:1 mixture of powdered limestone to braya mix and the last third into 100% braya mix. Each of these sets was further subdivided into one of two misting regimens, as the length of time needed for optimal survival was unknown. For the first misting subset the plants were placed under mist at 25-30°C for 2 weeks, as was done in other acclimatization studies (Amo-Marco and Lledo, 1996; Neuner and Beiderbeck, 1993). Then they were placed outside in a sunken bed for 2.5 months from July to October. For the second misting subset plants were kept under a mister at 25-30°C for 2 months and then placed outside in a sunken bed for 1 month.

Data Analyses

The General Linear Model (GLM) running on Minitab Statistical Software for Windows, Version 13.30 was used to carry out all statistical tests performed in this study. For each test residuals were examined for normality, independence, and homogeneity to

ensure that the statistical assumptions were not violated. The data were randomized if they were non-normal, as randomization tests make no assumptions (Schneider, 2003). However, if the sample size and the F-value were large then randomization was not done, as the General Linear Model is known to be robust to violations of assumptions (Schneider, 2003). P-values from these tests were used to determine whether differences between data were statistically significant when alpha was 5%.

Minitab was used to carry out general descriptive statistics. Sigma Plot for Windows, Version 3 was used to create all graphs and Microsoft Excel 2000 was used to organize all data.

Results and Discussion

Braya longii and Braya fernaldii

Germination of B. longii and B. fernaldii from seed was very successful in tissue culture media. Germination was between 53.6% for Burnt Cape disturbed, a B. fernaldii site, and 89.3% for Airport natural, a B. longii site, with 'site' being a significant factor ($F_{[3,113]}$=4.10, p=0.008 (randomized)) (Figure 4.1).

Site is also a significant factor with respect to the proportion of seedlings that were contaminated ($F_{[3,80]}$ =6.39, p=0.001 (randomized)); the highest contamination levels were associated with seeds from Burnt Cape Disturbed (BCD). The naturally growing plants at the BCD site have been monitored since the late 1990's and have been noted to display very uncharacteristic growth. Both the leaves and reproductive structures of the on-site plants appear fuzzy and noticeably different from those of plants growing on other sites. It is not currently known if this growth is due to a virus, fungus, or bacteria. However, both the proportion of contaminated cultured BCD plants and the markedly different yellow colour of the contaminated media from their tubes suggest some abnormality. Whether due to virus, fungus or bacteria this requires further investigation into the cause of the uncharacteristic plant growth.

As previously stated braya individuals were multiplied for the purpose of the acclimatization trial. However, while germination from seed was very successful, the explants multiplied by division of rosettes (Figure 4.2B) often failed to survive. In fact, approximately half of the explants multiplied in this way did not survive. The poor

survival is attributed to their inability to have leaves, roots and a viable meristem in every explant. Because there are no protocols for braya propagation, the plants may not have been of adequate size or had sufficiently developed roots to grow successfully. Symptoms could also indicate vitrification, which makes plants look water soaked with translucent leaves. In an *in vitro* study on the related species Brassica campestris ssp. chinensis var. utilis, the plant growth retardant triadimefon decreased vitrification as well as increased propagation and improved growth of seedlings (He et. al, 1998). The explants that were harvested from plantlets that initiated on healthy roots were more successful in tissue culture. However, their small size did not provide for a fast multiplication rate.

A total of 250 braya plants from tissue culture were multiplied in 6 months. They were divided into 3 different soil mixes and tested for their survival during the acclimatization process. After 10 days only 9.1% of all the braya had survived, after 4 weeks only 2% were alive and the last remaining plants died within 2 months (Figure 4.2C and 4.2D). Similar mortality rates were observed in all three soil mixes. This high mortality rate can largely be attributed to the condition of the plants when they were transferred from media to soil. Most of the plants were multiplied from rosettes and did not always have sufficient roots and their leaves were commonly brittle.

In many Brassicaceae studies successful growth in tissue culture requires experimentation with growth regulators (He et al., 1998; Souza et al., 1998). Any future tissue culture work done with braya should focus on media composition (specifically growth regulators) and multiplication before another acclimatization trial is attempted.

Salix jejuna

The genetic variation of S. jejuna in this study was largely uniform with all plant material originally from the Cape Norman A site. In 2002 several plants were harvested from this site and because of this there was enough suitable material to use in this study. The lack of interpopulational variation may have attributed to the uniformity of the explants; 50% of the nodal cuttings, which is how all multiplication was done, rooted within 1 week and 95% of all cuttings rooted within 2 weeks. Studies on related species have shown plant survival of 90% (Amo-Marco and Lledo, 1996) and 94% (Neuner and Beiderbeck, 1993) in some cases after transplanting. A study done with material from all available sites (a greater genetic difference) might show more variation (Neuner and Beiderbeck, 1993).

Within 6 months 500 plants had been established in media. This multiplication was so successful that the total number of plants could have been doubled if required. The survival of S. jejuna from tissue culture to acclimatization in soil was also very successful (Figure 4.3). In the first acclimatization trial, where plants were kept under a mister for only 2 weeks and then placed outside, the survival rate ranged from 67.5% in the braya mix to 94.8% in the 1:1 soil mix of braya mix to powdered limestone ($F_{[2,509]} = 38.33$, $p<0.001$ (randomized)).

The higher survival rate (over 85%) in the 1:1 and the 3:1 soil mixes (refer to Chapter 3 for soil information) in the first acclimatization trial is most likely due to the ability of the soil to retain moisture, giving the plants more time to establish in the soil.

The soil mixes that contained crushed limestone had a greater water holding capacity, which is crucial for survival in the first few weeks of acclimatization. In the second acclimatization trial the plants were kept under mist for a much greater amount of time, 2 months, before being transferred outside. And while a high humidity level is crucial for initial acclimatization, a prolonged time in such a humid, soil saturated, condition seemed to have had a negative affect on survival. As a result, the difference between the survival rates in the two acclimatization trials is significantly different ($F_{[1,509]}$ =42.83, $p<0.001$ (randomized)).

The differences can be attributed to the following: first, the survival rate is lower in all three soil mixes in the second acclimatization trial. The plants were quite often water-logged under the mister and the delicate balance between enough and too much mist played an important role in the higher amount of death observed. Second, the survival rate between the three soil mixes during the second acclimatization trial is significantly different than the one seen in the first acclimatization trial. The reason for this is not obvious and would need to be further studied. Overall, the acclimatization of S. jejuna was very successful and produced healthy plants (Figure 4.4C).

Once appropriate tissue culture protocols have been developed the focus of any future research done with endangered plants should be on acclimatization and the ability to re-introduce plants back into their native environment (Iriondo and Perez, 1990; Amo-Marco and Lledo, 1996; Kyte and Kleyn, 1996). It is very evident from this study that tissue culture is not the best method for increasing the *ex situ* population of the braya species. However, the *ex situ* braya plants are continuing to be grown successfully from

natural seed, which is why that method should be continued as the primary means of maintaining the *ex situ* populations of these two species.

Unlike the braya species, S. jejuna did exceptionally well in tissue culture and survival during acclimatization was as high as 94.8% (Figure 4.3) as it is with related species (Amo-Marco and Lledo, 1996; Neuner and Beiderbeck, 1993). These plants were very healthy and the results suggest that they would be suitable for re-introduction. Any future multiplication for experimental or re-introduction purposes should use plant material from all available sites in order to encompass a higher level of genetic variation. This material could be harvested from plants that are currently being maintained at the MUN Botanical Garden.

Recommendations

Maintaining explants in tissue culture is not required for either braya or S. jejuna. Maintaining them in tissue culture would only be required if the other methods for establishing *ex situ* populations (i.e. persistence of live plants or seed gene banking) were unsuccessful. As seen in Chapter 3 the *ex situ* populations of both braya and S. jejuna have been established and are thriving. However, if sterile plant material was required for laboratory experimentation then this technique would be very appropriate.

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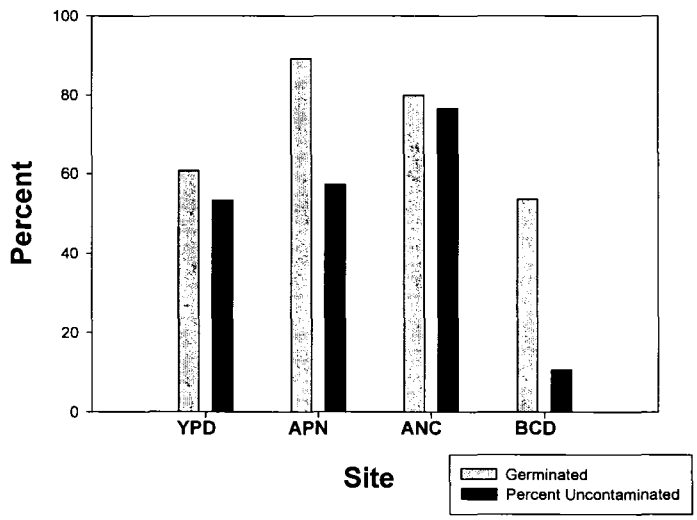

Figure 4.1: Percent germination of braya seed and percent uncontaminated in media. Sites included are YPD (Braya longii disturbed site), APN (B. longii natural site), ANC (B. fernaldii natural site) and BCD B. fernaldii disturbed site).

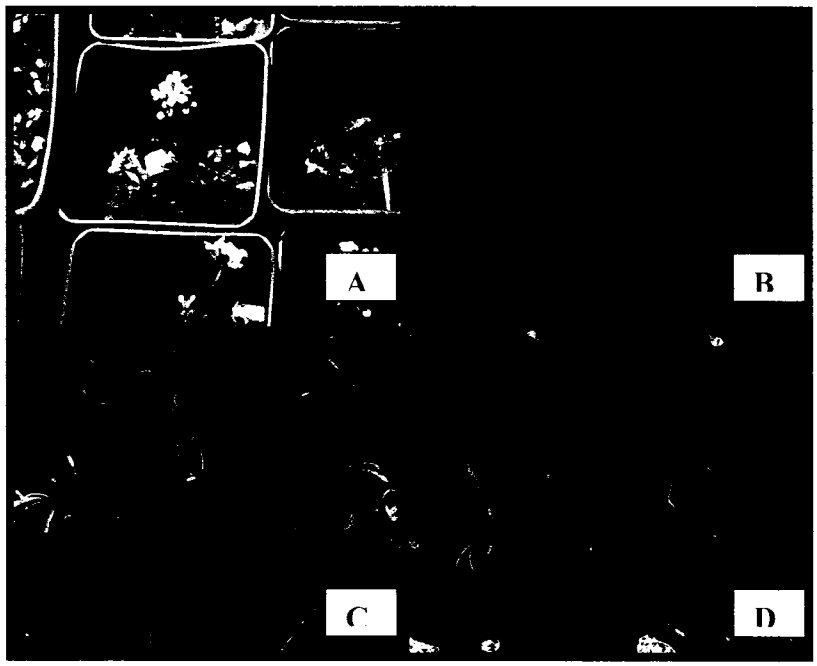

Figure 4.2: A) Two year old *ex situ* Braya longii started from seed growing at the MUN Botanical Garden. B) Braya growing in RLIBA rooting media. C) Braya transferred from media to soil (Day 1). D) Unsuccessfully acclimatized from media to soil (day 7).

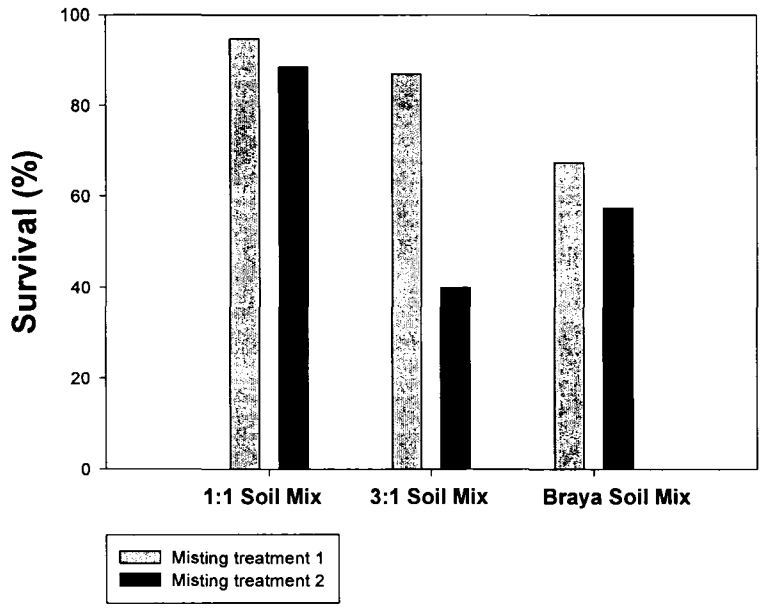

Figure 4.3: Survival of Salix jejuna from media to soil in both misting treatment 1 (plants were placed under mist for 2 weeks and then outside for 2 ½ months) and misting treatment 2 (plants were placed under mist for 2 months and then outside for 1 month). The 1:1 and 3:1 soil mixes refer to ratios of braya soil mix (an alpine-like mix) to crushed limestone.

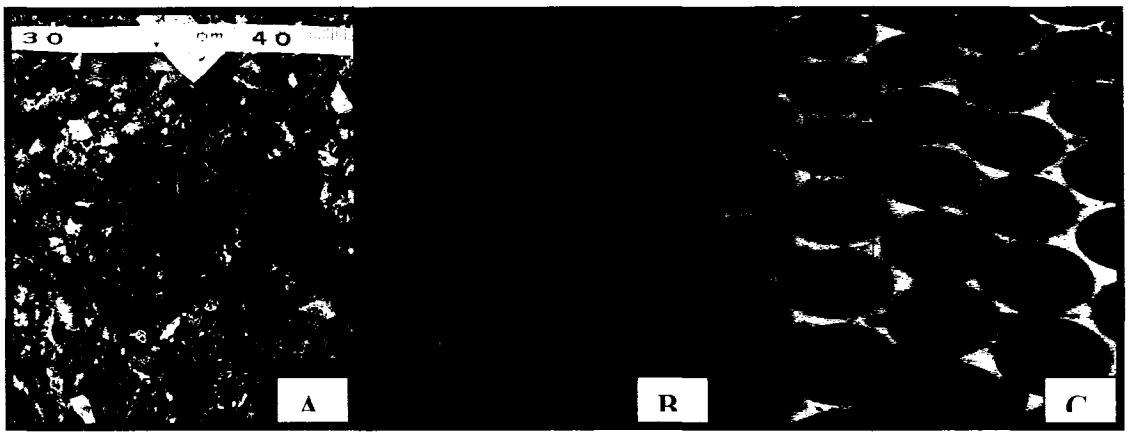

Figure 4.4: A) Naturally growing Salix jejuna from Cape Norman A site. B) Salix jejuna growing and rooting successfully in woody plant media (WPM). C) Successfully acclimatized S. jejuna after transfer from media to soil (1:1 soil mix in acclimatization trial #1).

Chapter 5. General Discussion

Ex situ Conservation Strategies

Persistence of live plants

Several methods are used in *ex situ* conservation to establish viable populations and maintain the genetic diversity of a species (Given, 1994). An important method is the development of protocols for the persistence of live plants. For both B. longii and B. fernaldii the focus on developing populations of live plants was through the optimization of a soil mix. The soil mix containing a 1:1 mix of braya mix (Appendix 2) to crushed limestone delivered desirable growth. The seeds sown in this mix grew into healthy plants that closely emulated the natural growth rate found in the *in situ* populations.

For Salix jejuna the focus on developing populations of live plants was on developing a protocol for the survival of cuttings. These cuttings grew exceptionally well and had a 69% survival rate. Developing a protocol for the persistence of live plants by means of sowing seeds in a suitable soil mix was not a focus of this project as the viability and longevity of S. jejuna seed was unknown at the beginning of this study.

Seed Gene Banking

Another method that is employed in *ex situ* conservation is the establishment of a seed gene bank. It had already been determined that both Braya longii and B. fernaldii seeds remain viable for several years if stored in a freezer at -20^{0}C (Hermanutz et al., 2002).

Originally Salix jejuna seed was assumed to have low longevity and viability like many willow species (Arya et al., 1988; Maroder et al., 2000), however the results of the germination study provided evidence that the seed is indeed viable for up to 9 months if stored in a freezer at -20^{0}C. In light of this a long-term seed gene bank may be possible with S. jejuna seed. A longer seed gene banking experiment would be required to confirm this.

Tissue Culture

The third method that is employed in *ex situ* conservation, and was utilized in this study, was the development of tissue culture protocols. The focus of this work was on the survival of both braya plants and S. jejuna from test tube to soil, the acclimatization step. The multiplication of both B. longii and B. fernaldii through tissue culture was not successful; further research should be conducted on the nutrient requirements of these plants in order to have a greater degree of success. In fact none of the plants survived longer than 1 month after transfer from test tube to soil (the acclimatization step).

The use of tissue culture to multiply S. jejuna was successful; survival rate was as high as 94.8% after acclimatization from test tube to soil. While the plants produced from tissue culture do not have a high degree of genetic diversity they may be used in further research thereby reducing the impact on natural sites.

Management Implications and Future Protocols

Braya longii and Braya fernaldii

First, it is imperative that a seed gene bank of these two species be maintained and replenished as necessary. Their natural populations produce a large amount of seed, so collecting enough for seed gene banking purposes will not further threaten them. By maintaining a seed gene bank the genetic diversity found in the wild can be preserved in the event that reintroduction is required.

However, as previously stated the protocols for growing these plants to maturity (persistence of live plants) are required in order for a seed gene bank to be valuable. As seen in Chapter 3 these species grew best in the 1:1 soil mix of braya mix to crushed limestone (Appendix 2); these plants were closer to the natural size and growth rate of braya found *in situ*. All future braya grown in the *ex situ* population should be grown in the 1:1 soil mix. Furthermore future management should consist of maintaining the present *ex situ* populations by transplanting braya into fresh 1:1 soil and larger pots as it becomes necessary.

As the *ex situ* populations of both B. longii and B. fernaldii appears to be thriving and stable the need for further tissue culture research is not essential to their survival. However if the need arises to study the developmental morphology of the species (either to determine any interpopulation or interspecies differences) or to increase the number of plants with a specific desirable trait (genotype) then tissue culture may indeed be a useful tool. However, nutrient requirements and successful acclimatization protocols would have to be determined first.

Salix jejuna

As seen in Chapter 3 the seed of S. jejuna is viable and the longevity is greater than originally suspected, therefore the opportunity to establish a long-term seed gene bank may indeed be possible. In the summer of 2005, the first of what should become an annual collection of S. jejuna seed was frozen at -20° C and stored. Annual collections are imperative as the seed may not remain viable if stored over a year. Germination success should be tested over a longer period of time than 9 months (which was the longest trial in this study) in order to determine how long the seed will remain viable. However, the failure of the seeds to establish on site (Chapter 2), despite the high viability of the seed indicates that seedling establishment on site is low. This should be investigated further in order to determine what effect, if any, this has on the survival of the species.

Monitoring of population density, growth rate, and reproductive capacity (both catkin production and seed production) on all currently established sites should continue in order to determine if the populations are stable. Salix jejuna is a long-lived plant, though the exact age structure is yet to be determined, therefore multiple years of data would be required in order to definitively state its population status, i.e. increasing, decreasing or stable.

The *ex situ* population of S. jejuna has been successfully established by means of cuttings from naturally growing plants on all currently established sites. These cuttings will need to be transferred into fresh braya mix and larger pots as necessary. Also the *ex situ* population should be replenished when plants die. The need for further research into

the establishment of an *ex situ* population of S. jejuna by means of seed is not crucial as the success of the cuttings is high.

Propagating S. jejuna from tissue culture was very successful and survival remained high through the final acclimatization stage. This remains a very useful tool to utilize if a large number of genetically similar individuals are needed, sterile explants are required for genetic or developmental analyses, or selected genotypes must be propagated for the purpose of re-introduction. Plants should be propagated in WPM, acclimatized as in the 1st acclimatization trial (Chapter 4) and transplanted to the 1:1 soil mix.

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Appendix 1: Recovery Objectives

Recovery objectives as stated in the National Recovery Plan for Long's braya (Braya longii Fernald) and Fernald's braya (Braya fernaldii Abbe) (Hermanutz et al., 2002):

1. Scientific Research
2. Population Monitoring
3. Critical Habitat Assessment and Protection
4. *Ex situ* Conservation
5. Education and Stewardship
6. Restoration and Species Reduction

Recovery objectives as stated in the Recovery Strategy for the Barrens Willow (Salix jejuna Fernald) (Djan-Chékar et al., 2003):

1. Assess and monitor the status of the natural population.
2. Define threats and limiting factor and mitigate controllable ones.
3. Lessen to the extent possible additional habitat loss and degradation due to human activities.
4. Encourage stewardship by the local residents.

Approaches to meet recovery objectives for Salix jejuna. Modified from the Recovery Strategy for the Barrens Willow (Salix jejuna Fernald) (Djan-Chékar et al., 2003).

Priority	Objectives	Actions
Urgent	1, 2 and 3	Biological surveys
Urgent	1, 2 and 3	Habitat protection
Urgent	1	Monitoring
Necessary	1	Demographic research
Necessary	1 and 3	Taxonomic research
Necessary	1, 2, 3 and 4	Ecological research
Necessary	4	Public outreach
Necessary	1, 2 and 3	Compliance to regulations
Beneficial	1	Genetic research
Beneficial	1 and 4	*Ex-situ* conservation
Beneficial	3 and 4	Restoration

Appendix 2: Protocols for Soil Development.

Alpine Mix: 5 parts topsoil, 2 parts leaf mulch and 1 part sand

Braya Mix: 10 parts topsoil to 1 part leaf mulch

1:1 Soil Mix: 1 part Braya mix to 1 part crushed and powdered limestone

3:1 Soil Mix: 3 parts Braya mix to 1 part crushed and powdered limestone

Particle size and nutrients for developed soil mixes.

	1:1 Soil Mix	**3:1 Soil Mix**	**Braya Mix**	**Alpine Mix**
% Silt/Clay (<0.0625 mm)	49.7%	38.0%	4.4%	5.9%
% Fine Sand (0.0625-0.25 mm)	19.2%	13.7%	7.2%	16.4%
% Medium Sand (0.25-0.5 mm)	6.7%	7.3%	12.4%	12.2%
% Coarse Sand (0.5-2 mm)	9.5%	16.9%	32.2%	24.4%
% Gravel (>2 mm)	14.9%	24.1%	43.8%	41.1%
pH	6.0	5.5	6.6	5.5
Potassium (ppm)	136	109	20	42
Phosphorus (ppm)	61	68	45	135
Calcium (ppm)	998	1087	1732	2854
Magnesium (ppm)	138	132	393	276
% Organic Matter	13.0%	NA	NA	NA

Appendix 3: Materials and methods used for soil analysis.

Soil collection

All soil cores were approximately 10 cm in length and 2.5 cm in diameter. Cores were taken near existing braya plants, but care was taken to ensure that there was minimal impact on the sites and that no braya were harmed during the process. Soil core were analyzed for pH, % nitrogen, calcium (ppm), phosphorus (ppm), potassium (ppm), magnesium (ppm), organic matter, particle size and moisture content. Not all samples could be analyzed for nutrients due to the expense.

In total 26 cores were taken in 2003 from: Port aux Choix, Anchor Point, Sandy Cove Airstrip, Green Island Cove, Burnt Cape, Sandy Cove Crusher (natural site), Sandy Cove Crusher (disturbed site) and Cape Norman.

In the summer of 2004, 160 soil samples were collected from all sites listed in Table 1.1 with the exclusion of YPN, WPD and ANC (St. Barb). Twenty-five of the 160 soil samples were taken from suitable but unoccupied sites. Due to the amount of large pebbles at some of the sites 10 cm x 2.5 cm soil cores were not taken; instead a small trowel was used to scoop up enough soil for analysis. In order to better understand the moisture content of the soil over the course of the summer, samples were taken in June, July, August and October. Moisture content was determined by weighing samples before and after drying, the difference was the % moisture content. In total 160 soil samples were taken in 2004: 59 samples in June, 26 samples in July, 24 samples in August and 51

samples in October. Along with these soil samples from the natural sites several artificial soil samples from the Botanical Garden (from past experimental attempts) were also analyzed for pH, nutrients and particle size.

The Soil and Feed Laboratory at Agriculture Canada analyzed select soil samples for pH, % N, Ca, P, K and Mg using the Mehlich III extraction method. Organic matter was determined by loss on ignition of samples in a muffle furnace. Textural analysis was done using a combination of dry and wet sieving techniques.

Particle size soil analysis

All soil samples were stored at 5 °C until they were analyzed. Particle size analysis was conducted by first using a standard wet-sieving protocol which determines the percent of silt and clay particles (< 0.0625 mm) in each sample (Allen, 1975). All samples were then dry sieved and the percent of fine sand (0.0625-0.25 mm), medium sand (0.25-0.5 mm), course sand (0.5-2 mm) and gravel (> 2 mm) was determined.

Organic matter analysis

Between 1 and 10 grams of dried soil per sample was used for organic matter analysis. The percent of organic matter per sample was determined by loss on ignition analysis as described by Berglund and Jasiewiczowa (1986). Particle size analysis could not be done on any samples that contained more than 50% organic matter.

Appendix 4: Soil nutrient analyses. See Appendix 3 for methods Nutrient analysis

Site	Species	Average Phosphorus (ppm)	Average Potassium (ppm)	Average Calcium (ppm)	Average Magnesium (ppm)
APN	BL	44	26	1916	758
SCN	BL	28	23	1912	385
SCD	BL	33	27	3220	935
YPD	BL	27	41	3785	125
CNN	BF	26	20	2176	358
ANC Pt.	BF	30	19	1542	430
BC	BF	16	25	5306	167
GIB	BF	31	25	3435	543
PAC	BF	21	18	4505	204

Appendix 5

Composition of McCown's Woody Plant Basal Salt Mixture (Lloyd and McCown, 1980)

Ingredient	mg/l
NH_4NO_3	400
$Ca(NO_3)_2 \cdot 4H_2O$	556
K_2SO_4	990
$CaCl_2 \cdot 2\ H_2O$	96
KH_2PO_4	170
H_3BO_3	6.2
$Na_2MoO_4 \cdot 2\ H_2O$	0.25
$MgSO_4 \cdot 7H_2O$	370
$MnSO_4 \cdot\ H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.6
$CuSO_4 \cdot 5H_2O$	0.25
$FeSO_4 \cdot 7H_2O$	27.8
$Na_2 \cdot$EDTA	37.3
Thiamine·HCl	1.0
Nicotinic acid	0.5
Pyridoxine·HCl	0.5
Glycine	2.0
Myo-inositol	100
Sucrose	20 g/l
Volume to	1000 ml
pH to	5.7
Agar	6 g/l

RLIBA Rooting Medium (Collins and Phillips 1982)

Ingredient	mg/l
Macronutrients	
NH_4NO_3	500
KNO_3	1050
KH_2PO_4	325
$NaH_2PO_4 \cdot H_2O$	43
$MgSO_4$	106
NaFeEDTA	25
$CaCl_2 \cdot 2H_2O$	600
Stock Solutions	
KI	0.5 ml
L2 Micronutrients	0.5 ml
L2 vitamins	0.5 ml
Nicotinic Acid	0.5 ml
Carbohydrate	
Inositol	125
Sucrose	15 g/l
Growth Regulators	
3-aminopyridine	2.5 ml
IBA	1.0 ml
Volume to	1000 ml
pH to	5.8
Agar	7.0 g

Stock Solutions (Pruski, 2004)

1. KI stock solution - 100mg/100 ml

100mg KI	100mg = 0.100 g
make to	100 ml with deionized, distilled water

2. L2 Micronutrients stock solution - 1000X

$MnSO_4 \cdot H_2O$	1.500 g
$ZnSO_4 \cdot 7H_2O$	0.500 g
H_3BO_3	0.500 g
$Na2MoO4 \cdot 2H_2O$	0.040 g

$CuSO_4 \cdot 5H_2O$	0.010 g
$CoCl2 \cdot 6H_2O$	0.010 g
Make to	100 ml with deionized distilled water

3. L2 Vitamins stock solution - 1000X

Thiamine·HCl	0.200 g
Pyridoxine·HCl	0.050 g
Make to	100 ml with deionized distilled water

4. Nicotinic Acid stock solution - 100 mg/100ml

100mg Nicotinic Acid	100mg = 0.100 g
make to	100ml with deionized, distilled water

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