Tolerance to sub-zero temperatures in *Phaseolus acutifolius* and interspecies hybrids between *Phaseolus vulgaris* and *P. acutifolius.*

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Abstract

Dry bean (*Phaseolus vulgaris*) is a sub-tropical crop severely affected by exposure to low temperatures during all of its growing stages. Cool spring temperatures and the risk of frost are major limiting factors for the early sowing of dry bean in Saskatchewan. Due to its economic importance; however, it has been introduced to Saskatchewan, but it needs to be made more cold tolerant to further expand acreage. The genes that can contribute some tolerance to low temperature stress in bean are not found within the primary gene pool, which limits the capability of breeders to generate a cultivar with such characteristics. Consequently studies have being done in order to find a possible source of genes that can induce tolerance to low temperature exposure. *Phaseolus acutifolius* is a relative of the domesticated dry bean and previous hybridizations with it have been successful. It is also known to be tolerant to abiotic stresses such as drought. For this reason the decision was taken to explore the level of resistance to low temperature stress exposure in several *P. acutifolius* accessions. Using whole plant freezing tests in controlled environment chambers, *P. acutifolius* W6 15578 was found to be more tolerant to exposure to sub-zero temperatures than were *P. vulgaris* genotypes. Interspecies hybrids were produced between *P. vulgaris* NY5-161 and W6 15578 and BC2 plants were produced using embryo rescue.

The whole plant freezing test is a destructive method that cannot be used with unique F_1 and BC_2 genotypes, so an alternative methodology to evaluate the hybrids was explored. An electrolyte leakage test was used and showed similar results to the whole plant freezing test with the parent plant controls. The F1 hybrids had an intermediate tolerance to low temperature stress and the further generations (BC1 and BC2) had a better level of tolerance to this kind of stress than the cultivated parent (NY5-161). This suggests that the genes that confer tolerance to low temperature exposure are being maintained through several generations of backcrossing and that these interspecies hybrids may offer a chance for the development of improved dry bean cultivars for the Saskatchewan environment.

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List of Abbreviations

ABA – abscisic acid

NPGS/USDA - National Plant Germplasm System, US Department of Agriculture.

BC - Backcross

P. ac 78 – *Phaseolus acutifolius* W6 15578

P. ac 45 - *Phaseolus acutifolius* PI 319445

P. ac 19 - *Phaseolus acutifolius* PI 430219

P. ang - *P. angustissimus* PI 535272

1. Introduction

The growing season in Saskatchewan is short and can be shortened further by late spring and early fall frosts. This climate has obliged breeders to develop varieties of different crops that are able to grow under these conditions. Common bean (*Phaseolus vulgaris*) is a crop of tropical origin and does not have any tolerance to sub-zero temperatures; consequently it is killed at ice formation temperatures (Balasubramanian et al., 2004). As a result, the regions it is generally adapted to are restricted. Because of its economic potential, however, it has been introduced to risky growing environments such as Saskatchewan.

Adaptation to this region has been improved through plant breeding, however sensitivity to chilling temperatures (low temperatures but above 0°C) and frost are still a problem. A wild relative of *P. vulgaris*, *P. angustissimus*, has been identified as being tolerant to a mild frost (Balasubramanian et al., 2004). *Phaseolus angustissimus*, or slimleaf bean, is native to northern Mexico and the southern Unites States. Balasubramanian (2004) demonstrated that it can survive at temperatures of -5°C to -7°C under field conditions. The ability to survive these sub-zero temperatures has lead researchers to focus on finding an effective way to transfer this low temperature tolerance into cultivated common bean. Hybrids between these two species have been developed (Belivanis and Dore, 1986; Balasubramanian 2002 and Schryer et al., 2005), but unfortunately the hybrids did not produce viable seeds and extensive backcrossing has been unsuccessful likely due to the genomic imbalance between these two species. As a result, new approaches to find and transfer genes that may confer tolerance to low temperatures in common bean are necessary.

Phaseolus acutifolius, is a closer relative to *P. vulgaris* than is *P. angustissimus* (Delgado-Salinas, 1999), and genes from it have been successfully transferred to common bean in the past (Scott and Michaels 1992). Previous studies have demonstrated that it is possible to generate introgression lines between *P. acutifolius* and *P. vulgaris* via embryo rescue (Andrade-Aguilar, 1988) and tolerance to common bacteria blight (CBB) has been successfully transferred to common bean (Scott and Michaels 1992). Balasubramanian (2004) demonstrated that the species *P. acutifolius* has some level of tolerance to sub-zero temperatures, but the lines were not as tolerant as *P. angustissimus*. Only a few accessions were evaluated in their study, which focused on accessions that were collected at high altitudes. Exposure to sub-zero temperatures

induces stress conditions that affect normal plant growth. Stress conditions can also be induced by other climatic conditions, such as heat and drought, to which *P. acutifolius* has been reported to have more tolerance (Markhart III 1985), suggesting that there may be other accessions with improved levels of cold tolerance.

The objectives of this study were to: (1) evaluate the tolerance to sub-zero temperature exposure of additional accessions of *P. acutifolius*, (2) identify an efficient methodology that will allow us to determine different levels of tolerance to exposure to sub-zero temperatures, and (3) asses the levels of tolerance in introgression lines developed from crosses between *P. vulgaris* and *P. acutifolius.*

2. Literature Review

2.1 Genus *Phaseolus*.

2.1.1 Common Bean (*Phaseolus vulgaris* L.)

Phaseolus vulgaris (common bean) is the most economically important species of the genus *Phaseolus*. It is part of the Fabaceae family, sub-family Faboideae, tribe Phaseoleae, subtribe Phaseolinae (USDA, 2007 & Debouck, 1991). There is not a specific center of origin, but rather two centers of domestication which are located in Mesoamerica (Mexico-central America) and in the Andean region of South America. Although it originated in these regions, today it is grown and consumed worldwide primarily due to its importance in human nutrition **(**Mercado-Ruaro and Delgado-Salinas, 2000*;* GRIN & NPGS 2007). There are two main forms of consumption of common bean in the world: fresh pods that are harvested and consumed at an immature stage, and dry beans. Dry beans occupy 85% of the worldwide production of common bean (Singh, 2001).

In Canada, common bean is not a new crop. It has been grown in small areas of the country since the middle of the 19th century. More recently, with worldwide population growth, the demand for this crop has increased, so new areas have been opened to this crop. In Canada the increase of cultivated area for common bean has been limited due to climatic constraints but it has been increasing in recent years due to the development of new cultivars that are more adapted to the northern latitudes.

Common bean plays an important nutritional role in many different regions of the world. It provides complementary protein to cereals. The nutritional profile of common bean is diverse, containing important macro- and micro-nutrients as well as dietary fiber and antioxidants. Some of these structural components play an important role in human health. For example, folate which is thought to reduce the risk of neural tube defects and the high content of dietary fiber helps to reduce blood cholesterol, it is abundant in common bean (Messina 1999).

2.1.2 Tepary bean (*Phaseolus acutifolius*)

Tepary bean (*Phaseolus acutifolius*) is one of the *Phaseolus* species that can be considered as a source of stress, pest and disease resistance that can be used to improve common bean

(Schinkel and Gepts 1988). This species has been grown in the southern U.S.A. and Mexico for approximately 8000 years. Originally a staple food, it is still consumed today like other dry beans. Tepary beans contain a high level of protein (23-25%) along with high concentration of minerals and other fatty acids (Hamama and Bhardwaj, 2002). Tepary bean grows wild in northern Mexico (Baja California, Chihuahua, Durango, Sinaloa and Sonora) and in the southwest U.S.A. (New Mexico, Texas and Arizona) where it is consumed locally. Recent studies have found that its distribution goes as far south as Costa Rica (Muñoz et al., 2006). Tepary bean in its cultivated form is found from 50 m to 1920 m in altitude. Its water requirements are low (150mm), but it also can be cultivated where precipitation is up to 750 mm (Debouck, 1991).

Physiological studies have been done to compare tepary bean with common bean; one of these studies evaluated the drought tolerance that exists in this species (Markhart III 1985). The *P. acutifolius* genotypes evaluated were classified as more tolerant to drought than the *P. vulgaris* genotypes and it was suggested that this may be because of specific physiological differences that exist. For example, much more sensitive stomates and a penetrating rooting system appeared to help delay dehydration in *P. acutifolius*. Other studies showed that *P. acutifolius* is also more heat tolerant compare with *P. vulgaris*. In a study done by Rainey and Griffiths, (2004), elevated temperatures (35°C day/32°C night) caused an 88% reduction in grain yield among the tepary beans while causing a 100% yield loss in the common beans studied.

Tepary bean does not only have some level of tolerance to abiotic stresses, but also tolerance to several biotic stresses. It has demonstrated levels of resistance to common bacteria blight which is a disease to which common bean is highly susceptible (Singh, 2001). Some resistant breeding lines were developed through interspecies hybridization between *P. vulgaris* and *P. acutifolius* (Scott & Michaels 1992; Singh and Muñoz 1999, Thomas and Waines 1984). These have since been used in the development of registered cultivars (e.g. OAC Rex; Michaels et al., 2008). This demonstrates that *P. acutifolius* is an important source of genes that can be use to make improvements on common bean.

Besides resistance to abiotic and biotic stresses, tepary bean has also been described as one of the healthiest foods for people with diabetes due to its low glycemic index (30), which is the ranking of carbohydrates their effect the levels of glucose in the blood, as well as it being an excellent source of fiber, vitamins, minerals and protein (Mendosa, 2006).

2.1.3 Slimleaf bean (*Phaseolus angustissimus*)

Phaseolus angustissimus is a wild relative of common bean that has been reported to be tolerant to exposure to sub-zero temperatures and was demonstrated to survive to -5°C to -7°C under field conditions and to -2.5°C under controlled conditions (Balasubramanian et al., 2004). Woronuk (2008) demonstrated that plants of this species managed to survive to temperature of -3.5°C while plants of *P. vulgaris* were all dead at -2.5°C. It has also been demonstrated to have some levels of tolerance to salinity (Bayuelo-Jimenez et al., 2002). It grows naturally in the southwest U.S.A. (New Mexico, Texas and Arizona) and in northern Mexico (Sonora) (Buhrow, 2007).

Hybridization between *P. vulgaris* and *P. angustissimus* was first reported by Belivanis and Doré, (1986) by rescuing zygotic embryos 13 to 23 days after pollination. Hybrid development between these two species was reported again by Balasubramanian, (2002). Despite extensive efforts to transfer these genes into common bean through backcrossing to these hybrids, it has not yet been possible, likely due to fertilization barriers and the genomic imbalance between these two species. Several attempts have been made to overcome the fertilization barriers through manipulation of media (Schryer et al., 2005) and the use of pollen manipulation techniques (Gurusamy et al., 2007) as well as the use of hormone injection or ovule culture methods (Gurusamy pers. comm.) but none of these techniques has worked for this combination of species.

2.2 Interspecific hybridization

The introgression of desirable genes into cultivated lines is an important process that is mainly done by breeders trying to overcome a specific problem. This is usually done by the use of other cultivated lines within the same gene pool. For many years plant breeders have used land races for crop improvement. Once breeders have explored all the alternatives within a species without success the next step is to try interspecific crosses. The development of interspecific hybrids usually results in fertility problems, typically generated by the genomic imbalance that exists between the two species that are being hybridized. The development of interspecific hybrids in *Phaseolus* usually requires the use of embryo rescue allowing the development of plants that can be used in breeding programs (e.g. Geerts et al., 1999). F_1 hybrids

usually have a high level of sterility which makes self pollination extremely difficult or impossible. It also makes it difficult to develop backcrossed hybrids. The incomplete chromosome pairing that occurs during meiosis in the F_1, caused by the genomic imbalance that exists between the two parental species and the resulting difficulty in recovering progeny, make this a difficult method to use in a breeding program.

Despite these problems, there have been many successes in transferring genes of interest from a wild species into cultivated beans. For example, genes related to tolerance to common bacteria blight have been transferred from *P. acutifolius* (CIAT, 1987, Scott and Michaels, 1992 and Thomas and Waines, 1984) and *P. coccineus* (Manshardt and Bassett, 1984) into *P. vulgaris* cultivated lines.

2.3 Plant stress

Plants can be greatly affected by both biotic and abiotic stresses. During any growing season plants are impacted by the attack of pests and diseases that can induce damage in the plant and thereby reduce yield, potential considerably. Water, temperature and nutrient deficiency induced stresses also have a tremendous impact on plant development and, ultimately, yield. In general, these factors can be controlled or the damage can be reduced by good agronomic management practices. These biotic and abiotic stresses that are affecting agricultural production worldwide also have an important economic impact due to the large numbers of products that need to be purchased and applied to reduce or control their effect. As a result, breeding for tolerance or resistance to these kinds of stresses is a high priority. In northerly regions there is an important abiotic stress that seriously affects agricultural production: exposure to low temperatures. Crops can be severely affected when exposed to chilling temperatures (Thomashow, 1998) and the agriculture industry loses billions of dollars every year due this problem. For example in the 1980's the cotton industry lost $60 million when cotton seedlings were affected by cold in the U.S. (Wilson, 1984). In the legume sector, even a light frost can cause serious losses in Canadian agriculture (Meyer and Badaruddin, 2001). Exposure to cold has multiple effects, both physiological and biochemical, on plant development especially in plants like beans, which are originally from tropical and subtropical areas.

2.3.1 Stress induced by cold temperatures

Cold stress is caused by exposure to low or sub-zero temperatures. Exposure to cold means that the plant will not be able to express its genetic potential which in turn will considerably impact yield outcomes. Cold temperatures have a significant impact on agricultural production in many area of the world; therefore increasing the levels of cold tolerance in cultivated plants is extremely important (Beck et al., 2004). Some of the major effects of exposure to low temperatures include loss of vigor, reduced growth, and flower abortion, all of which will affect final yield. Exposure of some species to low temperatures can even result in death of the plant. For example, tomato and pepper, two typically warm season crops, are highly susceptible to low temperature exposure. One example of a physiological change that is visible when tomato is exposed to low temperatures is the darkening of the leaf tissue which can represent cellular damage in the leaf that makes the plant more susceptible to attack by diseases due to the frost injury (LeBoeuf, 2004). In the case of pepper, it is even more susceptible to low temperatures exposure and can be killed when light frost occurs.

When plants are exposed to cold temperatures there are many physiological changes that can occur. These changes can be primary: ones that cause some malfunction in the plant, by reducing or changing some on the pathways in the plants, but these ones are not easily seen, while secondary changes are those generated upon exposure cold temperatures but, unlike primary injuries, these are visible like the present of dead tissue and irreversible (McKersie, 1994).

One of the main reasons a plant becomes stressed when exposed to sub-zero temperatures is due to the formation of extra-cellar ice which induces the cells to dehydrate (Chinnusamy and Zhu, 2007). In a simulated freezing test, damage is induced in the plant cell through exposure to low temperatures which results in failure of the cell membrane structure which in turn increases the membrane permeability allowing the electrolytes that are contain within the cell membrane to escape (Campos et al., 2002). A conductivity meter can then be used to evaluate the percentage of electrolyte leakage. This will allowed researchers to quantify the damage on the membrane which will hopefully correlate with the levels of tolerance that exist in the plant.

Some plants may increase their level of tolerance to freezing temperature exposure if they are exposure to a period of low but not freezing temperatures. This process can be referred as acclimation (Chinnusamy and Zhu, 2007). During the acclimation process the levels of soluble

sugars seem to increase functioning as an osmotic regulator reducing the dehydration of the cell, and preserving the integrity of the membrane (Yuanyuan et al., 2009). Many important crops, such as tomato, soybean and maize, however, do not acclimated and are therefore quite sensitive to cold stress (Chinnusamy and Zhu, 2007).

Besides the physiological changes that are induced by exposure to sub-zero temperatures, there are also many biochemical changes that occur at the same time. When plants are exposed to sub-optimal temperatures there can be a significant reduction in the photosynthetic rate (Leipner, 1998), the reduction on the photosynthetic rate can modify many functions within the plant. As a consequence, there is a reduction in enzyme activity in the plant (Byrd et al., 1995) which also generates a change in the protein content (Bredenkamp and Baker, 1994). There can also be a change in hormone levels, for example, ABA levels are increased when the plant is exposed to cold temperatures (Li, 1989). In contrast, gibberellic acid levels decrease when plants are acclimated (Li, 1989). All these changes can generate some damage to the plant, but these changes can also be induced as a defense mechanism to avoid injury. A good example is the physiological and biochemical changes that occurred to cotton plants during acclimation which are crucial for survival when exposed to low temperatures (Cottee et al., 2006). There have also been other studies were external application of hormones has been shown to have some affect on the level of tolerance to low temperature exposure. For example, in tomato, resistance to low temperature exposure is increased when 2-chloroethyl trimethylammonium chloride which is a plant growth substance is applied at a concentration of 200 ppm (Michniewicz and Kentzer ,1965). Other studies have also shown that external applications of ABA also increase the tolerance to exposure to low temperatures in some crops such as cucumber and cotton (Li, 1989), is possible to increase tolerance to cold temperatures through plant breeding. For example a cultivated genotype of *P. vulgaris*, NY5-161, has been developed at Cornell University and was shown to have a higher level of tolerance to cold exposure than other *Phaseolus* genotypes (Holobowicz and Dickson, 1989).

2.4 Evaluation of damage induced by low temperatures

To make the production of economically lucrative sub-tropical and tropical species possible in a harsh, northerly climate such as that of the Canadian prairies, breeding programs must focus on developing cultivars that are able to grow under low temperature stress. A basic

tool that is required for such a breeding program to succeed is the capability to screen plant material for variability in tolerance to low temperature stress.

Evaluating cultivars for chilling tolerance is not an easy task especially under field conditions. In the field, micro-climate variation is extremely high making it difficult to screen different cultivars for tolerance to exposure to sub-zero temperatures. It is also impossible to control the temperatures to which the plants are exposed so experiments can fail due to temperatures not being cold enough or being too cold to see differences among genotypes.

The damage induced by exposure to low temperatures is not only about the temperature to which the plants are exposed, but also the length of time that the plant is exposed to a given temperature. There are several options that breeders have been using to screen material for tolerance cold. One of them is the use of controlled environment chambers; the point of these experiments is to control the environment in which plants are going to be grown and evaluated. In these chambers, researchers are able to control the temperature at which they would like to expose the plants, which allows them to be able to evaluate specific parameters. Temperature can be reduce gradually and maintained for set lengths of time, which allowed researchers to take samples at different temperatures. Balasubramanian, (2002) used these chambers to evaluate the levels of tolerance to sub-zero temperatures of several *Phaseolus* species, like *P. filiformis, P. angustissimus, P. ritensis* and *P. acutifolius*. In a follow-up study, he also used open field trials to confirm the chamber results. Woronuk, (2008) also evaluated *P. vulgaris* and *P. angustissimus* at different sub-zero temperatures in a controlled growth cabinet. The problem with these tests is the restricted space available in the growth chambers limited the number of samples can be tested at a given time. Other methodologies have being used to determinate the low temperature tolerance, Balasubramanian, (2002) used detached leaflets, exposing them to low temperatures in a controlled environment chamber and then measured the electrolyte leakage ratio to estimate survival.

Another option is the use of an electrolyte leakage test following exposure of leaf discs, placed in glass tubes, to sub-zero temperatures in a cryo-bath. After the treatment, the percentage of electrolyte leakage ratio is determined as explain by Balasubramanian (2002). This methodology has also been use to evaluated freezing tolerance in several crops such as sugarcane (Miller et al., 2004), rose clover (Nunes et al., 2003), cotton (Cottee et al., 2006), *Coffea* sp. (Campos et al., 2002), and maize (Szalai et al., 1996). These studies showed that

electrolyte leakage can be an effective methodology for detecting different levels of freezing tolerance for commercial crops. In the study done by Cottee et al., (2006) they showed that the cotton cultivar Namcala was more tolerant to exposure to cold temperatures than was the cultivar DP16, which was comparable to the results that they obtained using the whole plant freezing tests.

3. Materials and Methods

3.1. Plant material

All genotypes used in this study and their sources are listed in Table 3.1. Two cultivated *Phaseolus vulgaris* L. lines were used: ICA Pijao and NY5-161. ICA Pijao is a line used previously for interspecific hybrid development at the International Center for Tropical Agriculture (CIAT) in Colombia and has been demonstrated to combine well when hybridized with other *Phaseolus* species (Mejia-Jimenez et al., 1994). It is of sub-tropical origin, and it is highly susceptible to damage when exposed to low temperatures (Woronuk, 2008). It is not well adapted to Saskatchewan growing conditions. NY5-161 is a breeding line developed by M. Dickson at Cornell University in New York State that has been shown to have some improved tolerance to low but non-freezing temperatures over other common bean lines (Dickson and Boettger, 1984). No previous attempts to use this genotype in interspecific crosses had been tried and its success in an interspecies crossing program was unknown.

The wild species of the genus *Phaseolus* that were tested included *P. acutifolius* spp. *acutifolius*, *P. acutifolius* spp. *tenuifolius* and *P. angustissimus*. Previous studies have shown that the *P. angustissimus* accession used is more tolerant than *P. vulgaris* and is able to survive the exposure to temperatures as low as -5°C and -7°C under field conditions (Balasubramanian et al., 2004).

Table 3.1. Description of plant material used for the experiments

Species	**Genotype**	**Location**	**Source**	**Improvement status**
Phaseolus vulgaris	ICA Pijao	Colombia	Colombia, CIAT	Cultivar
P. vulgaris	NY5-161	New York, USA	Cornell Univ.	Breeding line
P. acutifolius spp. *acutifolius*	PI 319445	Baja Norte, Mexico	USDA-ARS*	Wild material
P. acutifolius spp. *acutifolius*	W6 15578	Mexico	USDA-ARS *	Wild material
P. acutifolius spp. *tenuifolius*	PI 430219	New Mexico, USA	USDA-ARS *	Landrace
P. acutifolius spp. *tenuifolius*	W6 20127	Chihuahua, Mexico	USDA-ARS *	Wild material
P. angustissimus	PI 535272	New Mexico, USA	USDA-ARS *	Wild material

* USDA-ARS Western Regional Plant Introduction Station, Pullman, Washington.

Three BC_1 hybrids were developed from crosses between NY5-161 and W6 15578 backcrossed to NY5-161 for use in this project. These generations required embryo rescue to survive (Gurusamy pers. comm.). Cuttings of the F_1 and BC_1 plants were obtained and maintained through cuttings for testing. The BC_1 plants were backcrossed to NY5-161 one more time to develop six BC_2 individuals (Fig. 3.1) also through the use of embryo rescue techniques. The media used was an enriched basal medium discover by Schryer et al., (2005) where they used to support embryo development.

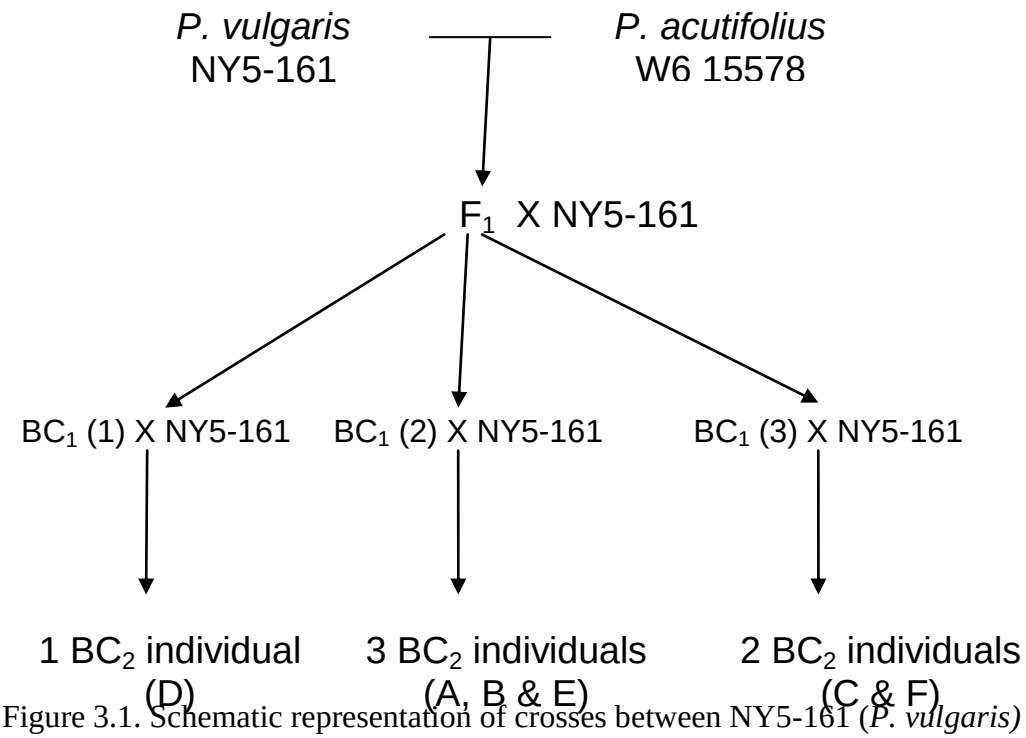

Figure 3.1. Schematic representation of crosses between NY5-161 (*P. vulgaris)* and W6 15578 (*P. acutifolius*) to develop F_1, BC_1 and BC_2 interspecific hybrids.

3.1.1 Pollen viability test

To evaluate the pollen viability of the different interspecies hybrids and progeny, anthers obtained from flowers one day prior to opening were collected from each genotype three separate times during a period of one week. A drop of acetocarmine staining solution (1%) was added to the pollen grains after they were extracted from the anthers. Counts of stained and unstained pollen grains were made using an optical microscope (20X). The percentage of viable pollen (stained) was calculated by comparing it with the total number of pollen grains in the sample (Mamatha et al., 1993).

3.2. Screening protocols for assessing tolerance to sub-zero temperatures

3.2.1. Whole plant exposure in controlled environment chambers

Seed coats were nicked with a sharp blade and placed in a Petri dish (100mm x 15mm; VWR International) on wet filter paper (11.0 cm; Whatman, Maidstone UK) to germinate. Germinated seeds were transplanted to Styrofoam boxes (24.1x16.5x15.2 cm) filled with Redi-Earth soilless mix (WR Grace, Ajax Ontario) and a layer of pea gravel in the bottom for drainage. Plants were grown in a controlled environment chamber (model PGV36, Conviron, Winnipeg, MB) set to 23°C (day) and 18°C (night), with a 12 hr photoperiod and a light intensity of 230 µmol $m^{-2}s^{-1}$. Plants were watered as needed. Three weeks after transplanting, the plants were exposed to low, but non-freezing temperatures (7°C day and 5°C night) for 72 hrs with a 12 hr photoperiod (Balasubramanian, 2002). To prevent the top layer of growing media from freezing during the freezing experiment, a thin layer of dry Redi Earth soilless mix was applied to the surface.

The freezing test was started by moving the plants to a growth chamber capable of reaching sub-zero temperatures. The cabinet temperature was initially decreased from 5°C to -1°C and the lights were left off since light has a direct effect on cold hardiness (Nijjar and Sites, 1959). The temperature inside the chamber was monitored using a HOBO PRO data logger (OnSet, Bourne MA). Once the temperature stabilized, ice nucleation was induced by spraying a fine layer of atomized water over the plants. The temperature was decreased 1°C per hour until the desired first temperature point was reached. The initial temperature varied according to individual experiment. Once the desired starting temperature had been reached it was held for one hour prior to sampling and then the temperature was again reduced. This procedure was repeated until samples had been taken at all pre-determined temperatures. Sampled plants were removed to a cabinet set to 5°C dark and 7°C light. The survival data were collected from the samples 12 hrs after the total sampling was completed.

3.2.2. Electrolyte leakage test for assessing tolerance to sub-zero temperatures

A total of 10 seeds each of each genotype tested were nicked to encourage better germination. Germinated seeds were transferred to 25 cm pots filled with Redi-Earth. Seeds of BC_2 hybrids were similarly treated and transferred to Sunshine Mix #3 (Sun Gro Horticulture, Vancouver, BC) after germination. Once the plants were well established, cuttings of two nodes were taken, dipped in a powdered rooting hormone (0.1% IBA, manufacturer at Chattanooga, TN) and transferred to Sunshine Mix #3. Cuttings were fully covered with plastic cups for five days to maximize relative humidity, then the cover was

remove gradually to allow plants to adapt to the normal environment. Cuttings of BC_1 and F_1 plants were similarly produced. Plants generated from seed and from cuttings were grown until they had fully extended healthy leaves on still vegetative plants. Then one or two leaves were taken from the plants and leaf discs were extracted using a 1 cm diameter cork borer. The leaf discs were washed with ddH_2O to reduce leakage from the cut edges which could affect the results of the experiment. Single discs were placed in individual test tubes that were labeled with genotype and temperature. The test tubes with the samples were placed in a glyco bath (LT 50 DD circulating bath, Neslab Instrument Inc, Newington NH) set to -2 °C. The samples were left at -2°C for two hours to equilibrate. Then ice nucleation was by placing ice crystals directly on the tissue. Immediately following ice nucleation, the temperature was decreased to -2.5°C and left for 1hr. After one hour, sub-samples of each genotype were removed from the bath and placed at in a cold chamber set at 8°C. Then the temperature of the glyco bath was decreased to -3.0°C and left there for 1 hr before sub-samples were taken again. The same procedure was repeated at -3.5°C and -4.0°C. After 12 hrs at 8°C, the samples were removed from the cold chamber and the leaf samples were taken out of the tubes and transferred to 125 ml Erlenmeyer flasks. A total of 10 ml of ddH_2O was added and the samples were agitated for 5 hrs at room temperature on a rotary shaker. A conductivity measurement was done for each sample using a YSI model 35 conductivity meter (Yellow Springs Instrument Co. Inc. Ohio) with the sensitivity set at 200 mho. After the data were collected, the samples were frozen at -80°C for 24 hrs. After thawing and agitating for 5 hrs at room temperature, a final conductivity reading was taken and the percentage retention of electrolytes was determined by subtracting the initial conductivity reading from the final reading.

Due to space restrictions in the glycol bath, two sets of experiments were conducted. In the first set, seven different genotypes were evaluated using an RCBD with three repetitions (ICA Pijao, NY5-161, *P. acutifolius* W6 15578, *P. acutifolius* PI 430219, *P. angustissimus,* F_1-1, and BC_1-1). In the second set of experiments, the six BC_2 genotypes and two parents, NY5-161 and W6 15578, were evaluated using an RCBD with three repetitions.

3.3 Statistical analysis

The software used for the all analyses was The SAS System 8.2 (SAS Institute Inc., Cary, North Carolina, USA, 1985). For the pollen viability test (section 4.1.2), the results were compared using a Fisher's Protected LSD test with a $P \leq 0.05$ significance level, using Proc GLM.

Due to the small number of plants that were use in the first set of the whole plant freezing experiments, the statistical analyses were done using a log-linear model (G-test) following the procedure used by Lamb et al. (2007) to determine if there were statistical differences among the genotypes. This log-linear model considers the number of surviving plants per each combination of treatments as the response variable for Poisson distributed data.

The set of experiment where there was a comparison between acclimated plants vs. not acclimated (Section 4.3) and the unifoliate plant vs. the third-trifoliate plants (Section 4.4) was analyzed using a simple t-test to determine if there were significant differences among the treatments for each of the genotypes.

Electrolyte leakage data were subjected to an analysis of variance (ANOVA) and protocol and treatments means were compared using Fisher's Protected LSD test with a P<=0.05 significance level, using a GLM model .

4. Results

4.1. Hybrid characterization

4.1.1. Hybrid morphology and observations

In this study a general morphological characterization of the parents, as well as the F_1, BC_1 and BC_2 hybrids was done. The morphology of the hybrids will be described relative to the morphology of the parental lines.

NY5-161 was used as the female parent in the production of interspecific hybrids. It has large white flowers with a tinge of light pink on the petals. The pedicel that joins the flowers to the plant is short and green, the leaves of the plant are large, and the plants have a determinate growth habit. This genotype seems to be highly susceptible to drought with considerable wilting evident when left un-watered for too long. The seed color of NY5-161 is a uniform brown.

W6 15578 was the *P. acutifolius* genotype used as the male parent in the interspecies crosses. It has small flowers with a strong pink color. The pedicel that joins the flower to the plant is medium- long with a distinctive green and red color, and the plants have an indeterminate growth habit. The leaves of W6 15578 are small compared to those of NY5-161 and the plants seem less affected by drought compared to NY5-161. The seed coats of this genotype have a darker brown color than does NY5-161 and black spots are distributed across the seed coat.

Based on the morphological characteristics of the F_1 (NY5-161 X W6 15578) it was clearly a hybrid between the two parents. It had small, pink-colored flowers. The pedicel was as short as that of NY5-161, but it showed the green and red color present in W6 15578. The hybrid plant had an indeterminate growth habit but seemed to be weaker and had less branching than the parents. The leaves of the hybrid plants were similar to those of W6 15578. The hybrid seemed to have increased levels of drought tolerance over the NY5-161 parent. It was not possible to obtain seed from the F_1 due to the high level of sterility present in the reproductive organs.

Three BC_1 hybrids were developed by crossing the F_1 with NY5-161 and rescuing the embryos. The first one, BC_1-1, had large, light pink flowers with pedicels that were short and green like NY5-161. That plant had an indeterminate growth habit with normal growth and branching. The leaves were bigger that those of the F_1 but still smaller than those of NY5-161. It seemed to be drought susceptible like NY5-161. The plant did not produce any seed.

BC_1-2 had smaller flowers with a strong pink color and long green and red pedicels. The plant had indeterminate growth habit, with normal growth and branching. The leaves were bigger than those of the F_1 but still smaller than NY5-161. It seemed to be drought susceptible like the other BC_1. This plant also failed to produce seed.

BC_1-3 had large flowers with a strong pink color, and the pedicels were short with green and red coloration. This plant had an indeterminate growth habit with a reduced level of branching. The leaves were bigger that those of the F_1 but still smaller than NY5-161. It seemed to have better drought tolerance than NY5-161. Once again, seed was not produced by the plant.

A total of six BC_2 hybrids were derived from three BC_1 plants by backcrossing to NY5-161 again. Their morphological characteristics are described in Table 4.1 and seed characteristics are shown in Figure 4.1.

Table 4.1. Morphological characteristics of six different BC_2 plants derived from a backcross program using NY5-161 as the recurrent parent.

Genotype	**Growth habit**	**Flower**	**Leaf size**	**Pedicel**	**Seed Coat Color of BC_3 seeds**
NY5-161	Determinate	Big white flower	Big leaf	Short and green	Brown
W6 15578 (P.ac 78)	Indeterminate	Small pink flower	Small leaf	Medium to large with red and green	Dark brown with darker spots
BC2–A (BC1-2xNY5-161)	Indeterminate	Light pink, big flower	Bigger than P.ac78 but smaller that NY5-161	Medium size, green with red color	Black
BC2–B (BC1-2xNY5-161)	Determinate	Big white flower	Leaves size was similar to NY5-161	Short with green color	Brown
BC2–C (BC1-3xNY5-161)	Determinate	Big flowers with a light pink color	Bigger than P.ac78 but smaller that NY5-161	Large with green color	Dark brown, with lighter spots
BC2–D (BC1-1xNY5-161)	Determinate	Big flowers with a light pink color	Bigger than P.ac78 but smaller that NY5-161	Short with green and red color	Black
BC2–E (BC1-2xNY5-161)	Indeterminate	Big flowers with a pink color	Bigger than P.ac78 but smaller that NY5-161	Short with green color	Dark brown with lighter spots
BC2–F (BC1-3xNY5-161)	Determinate	Big flowers with a white color similar to NY5-161, except for a tinge of pink.	Leaves were similar to the NY5-161	Large with green color	Dark brown with lighter spots

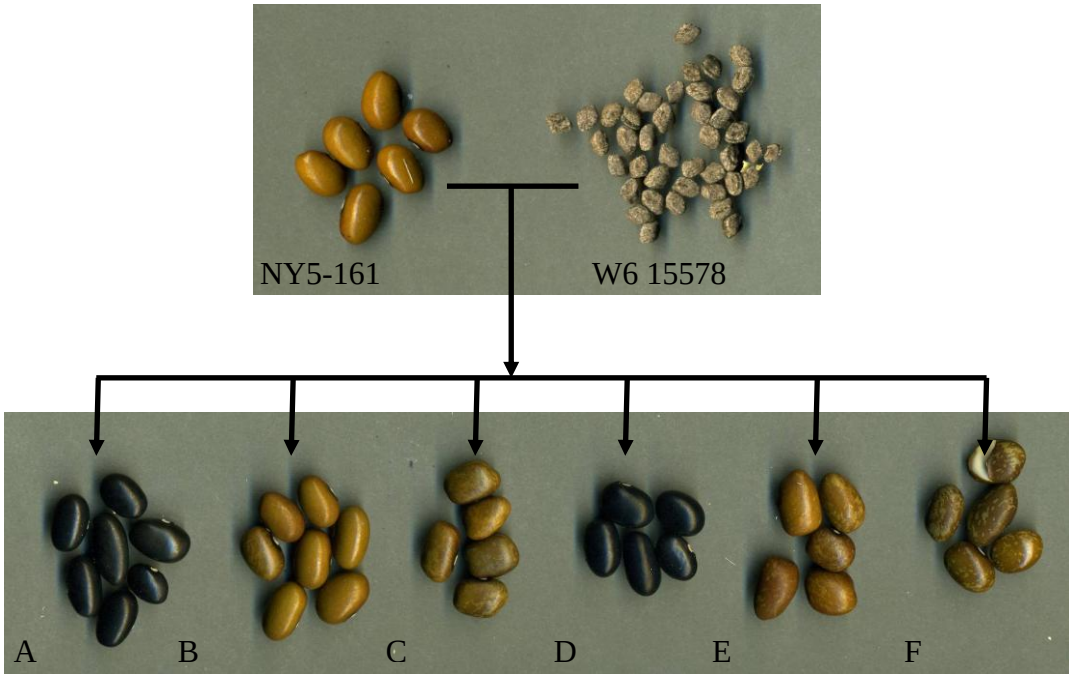

Figure 4.1. Seed coat color segregation in the BC_3 generation (BC_2 seed coats).

4.1.2. Pollen Viability test

A pollen viability test was done on the different genotypes that were available. Only one of the three BC_1 was still alive at the time of testing. The results of this test showed that there was a highly significant difference in percent pollen viability amongst genotypes that were developed ($P < 0.0001$; Fig. 4.2). The F_1 hybrid had the lowest percentage of pollen viability. The BC_1 had a higher percentage of pollen viability than the F_1 hybrid. The BC_2 plants had a considerable increase in pollen viability compared to the F_1 hybrid and the BC_1. BC_2-B was the most fertile, having 96% pollen viability, which was no different than the parents ($P > 0.05$). Four BC_2 F_1 plants (A, C, E and F) had significantly lower pollen viability than BC_2-B ($P < 0.05$). BC_2-D had the lowest pollen viability at 68%; it was lower than the other five BC_2 plants ($P<0.05$), yet still higher than the F_1 and BC_1 ($P < 0.05$).

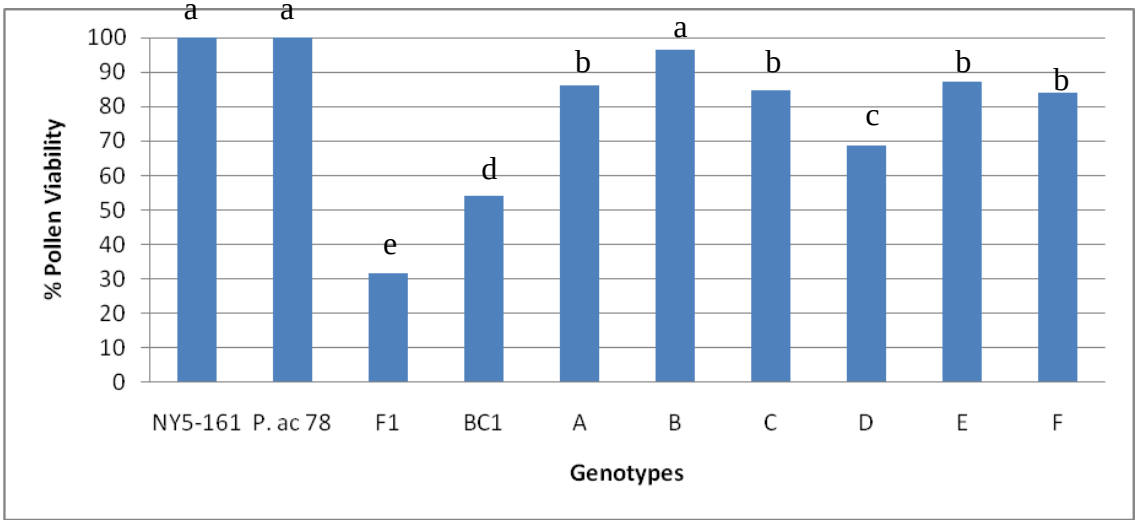

Figure 4.2. Percent pollen viability of parents (*P. vulgaris* NY5-161 and *P. acutifolius* W6 15578 (P.ac 78)), interspecific F_1 hybrid (NY5-161 X W6 15578), BC_1 (F_1 X NY5-161), and six BC_2 progeny (BC_1 X NY5-161; labeled A through F) grown under standard growing conditions. Bars with the same letter represent genotypes that are not statistically different from one another ($p>0.05$).

4.2 Survival of whole plants following exposure to sub-zero temperatures

In a one-replicate experiment, seven accessions, representing three different species (Table 4.2), were exposed to decreasing temperatures and assessed for percent survival at -2 to -3 °C. There were marked differences among the species and within the species for survival at these temperatures (Table 4.2). *Phaseolus vulgaris* line ICA Pijao was the least tolerant having only 25% survival at -2°C and 0% at -2.5°C and -3.0°C. The other *P.* vulgaris line, NY5-161, which is reportedly more cold tolerant (Holobowicz and Dickson, 1989), had 50% survival at – 2°C. Like ICA Pijao, however, it did not survive at -2.5°C or at -3.0°C.

The *P. angustissimus* line, as well as the *P. acutifolius* lines, showed some improved tolerance to sub-zero temperatures (Table 4.2). The four *P. acutifolius* lines that were tested showed different levels of tolerance to sub-zero temperatures. The *P. acutifolius* spp. *tenuifolius* lines, PI 430219 and W6 20127, had only 25% survival at -2.5°C and neither survived exposure to -3.0°C (Table 4.2). In contrast, the two *P. acutifolius* spp. *acutifolius* lines, W6 15578 and PI 319445, demonstrated a higher tolerance to sub-zero temperatures, with more than 50% and 25% survival, respectively, at -3.0°C.

Table 4.2. Percentage survival of seven different *Phaseolus* genotypes following step-wise exposure to -2°C, -2.5°C and -3°C for 1 hour each. Data obtain from one repetition of four plants per temperature.

Genotype	Species	% survival at		
		-2.0°C	-2.5°C	-3°C
ICA Pijao	*P. vulgaris*	25	0	0
NY5-161	*P. vulgaris*	50	0	0
PI 535272	*P. angustissimus*	100	50	25
W6 15578	*P. acutifolius spp. acutifolius*	100	75	50
PI 319445	*P. acutifolius spp. acutifolius*	75	50	25
PI 430219	*P. acutifolius spp. tenuifolius*	75	25	0
W6 20127	*P. acutifolius spp. tenuifolius*	50	25	0

Based on these initial results, a smaller set of genotypes (Fig. 4.3) were selected for further study with the objective to focus on the tolerance levels in the *P. acutifolius* lines and increase the number of replications to be more confident in the results. The temperatures surveyed were changed to -3.0°C, -3.5°C, and -4.0°C. PI 535272 (*P. angustissimus*), plus ICA Pijao (*P. vulgaris*) were used as tolerant and susceptible checks, respectively, based on results of experiment one and previous reports (Balasubramanian et al., 2004).

Only a few ICA Pijao plants survived at a temperature of -3.0°C; significantly fewer than W6 15578 ($p=0.027$) but not significantly different from PI 535272 ($p=0.199$) and PI 319445 ($p=0.124$). When tested at lower temperatures none of the ICA Pijao plants survived unlike the other lines (Fig 4.3). *Phaseolus angustissimus* PI 535272 continued to show better levels of tolerance with 50% survival at -3.5°C, and 33% of the plants survived exposure -4.0°C.

The two *P. acutifolius* lines (W6 15578 and PI 319445) that were selected based on the results of the first experiment, showed levels of tolerance similar to *P. angustissimus* PI 535272. In this experiment W6 15578 had 58% survival at -4.0°C; significantly different from ICA Pijao but not significantly different from PI 319445 and PI 535272. The other *P. acutifolius* line, PI 319445, had 42% survival at -4.0°C (Fig 4.3). The statistical analyses

indicated that all the three genotypes from the wild species were not significantly different from one another in terms of survival (P>0.05 at all temperatures).

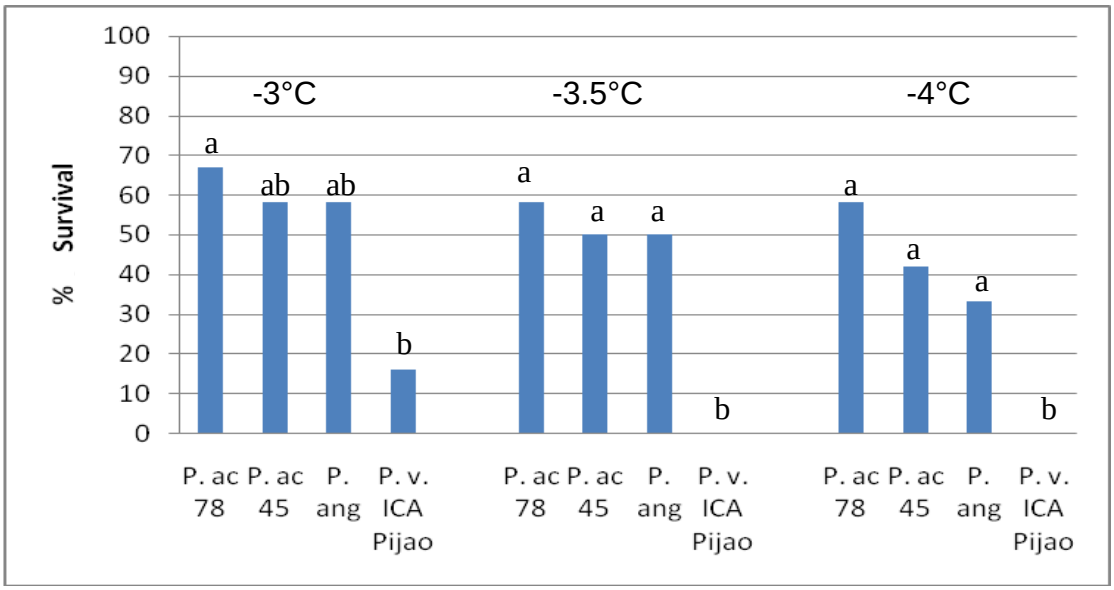

Fig 4.3. Percentage survival of *P. acutifolius* W6 15578 (P.ac 78), *P. acutifolius* PI 319445 (P.ac 45), *P. angustissimus* PI 535272 (P. ang), *P. vulgaris* (P.v ICA Pijao) following exposure to -3°C, -3.5 °C and -4.0°C for 1 hour each. Means of three replications of four plants each. Bars with the same letter represent genotypes that are not statistically different from one another (p>0.05).

To obtain a more accurate data of the levels of tolerance of the *P. acutifolius* genotypes another experiment was set up using only *P. acutifolius* accessions but with larger numbers of plants per sample. The ICA Pijao plants that were placed in the chamber to verify that freezing conditions occurred all died.

The three different *P. acutifolius* genotypes had different levels of tolerance to the sub-zero temperatures surveyed at -2.5°C and -3.5°C. When the accessions were exposed to -4.0°C, the percentage of survival of all accessions decreased considerably, with only one or two plants of each surviving. There was no significant difference in survival among the genotypes at this temperature (p>0.05). While there appeared to be a difference in survival among the genotypes at -3.0°C, it was not statistically different (Fig. 4.4; P>0.05).

W6 15578 appeared to be the most tolerant of the three genotypes evaluated (Fig. 4.4). In this larger sample size experiment, W6 15578 had 90% survival when exposed to -2.5 °C, significantly better (p= 0.016) than PI 430219. This line also had 52% survival when exposed to -3.0°C; and 48% survival at -3.5°C. At -3.5°C there were significantly more

plants of this accession alive than for the other two lines (p=0.003). PI 319445 had 72% survival when expose to -2.5°C, which was less than W6 15578 at the same temperature and time of exposure but not statistically different. When the temperature was decreased to -3.0 °C, PI 319445 showed only a 34% survival rate, and when exposed to -3.5°C it was significantly less tolerant than W6 15578 (p=0.003).

The other accession included in the experiment was PI 430219 which in previous experiments had been more susceptible than W6 15578 and PI 319445. In this experiment it only had 50% survival when exposed to -2.5°C significantly less than W6 15578. It was similarly less tolerant at -3.5°C (Fig. 4.4).

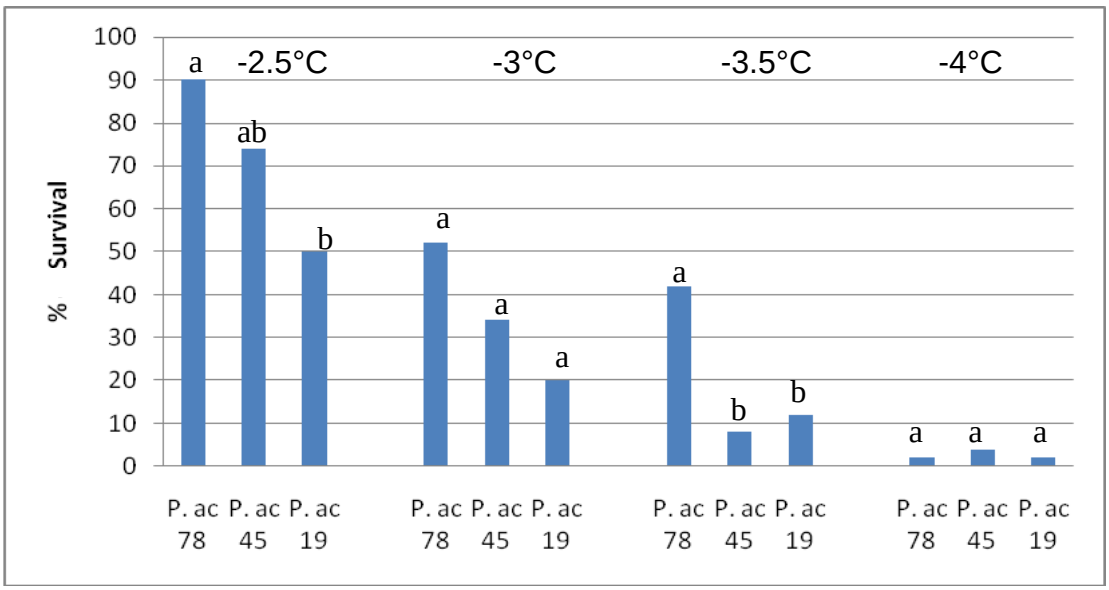

Figure 4.4. Percent survival of *P. acutifolius* W6 15578 (P.ac 78), *P. acutifolius* PI 319445 (P. ac 45), and *P. acutifolius* PI 430219 (P. ac 19) following step-wise exposure to -2.5°C, -3.0°C, -3.5°C, and -4°C, for 1 hour each. Means of two replications of 25 plants each. Letters above bars represent significant differences among the genotypes using a G-test.

4.3. Response to acclimation conditions

When W6 15578 and PI 430219 were exposed to sub-zero temperatures, there was no significant difference in the level of tolerance between plants that were acclimated vs. the ones that were not at any of the temperatures surveyed except at -3.5°C where non-acclimated plants of W6 15578 had better survival than the acclimated ones ($P<0.05$).

Table 4.3. Percentage survival of 2 (*P. acutifolius* W6 15578 (P. ac 78), and *P. acutifolius* PI 430219 (P. ac 19) following exposure to -2.5°C, -3.0°C, -3.5°C, with (A) and without (NA) a 3 day acclimation period. Means of two replications of 25 plants each.

Temperature	-2.5°C		-3.0°C		-3.5°C	
Treatment / Genotype	A	NA	A	NA	A	NA
P.ac 78	90	78	52	72	42*	58*
P.ac 19	50	64	20	48	12	12

* represents significant difference between A and NA treatments (P<0.05).

4.4. The effect of plant growth stage on tolerance to sub-zero temperatures

When W6 15578 plants were exposed to -2.5°C, survival at the third-trifoliate stage was significantly higher than when the same accession was evaluated at the unifoliate stage (Table 4.4; p=0.02). At all other temperatures, there was no significant difference in survival rate (P>0.05). There were no significant differences in survival between the unifoliate and third-trifoliate plants of the genotype PI 430219 (P>0.05).

Table 4.4. Percentage survival of *P. acutifolius* W6 15578 (P. ac 78), and *P. acutifolius* PI 430219 (P. ac 19) following exposure to -2.5°C, -3.0°C, -3.5°C, at the third-trifoliate (Trifol) and unifoliate (Unifol) stage. Means of two replications of 25 plants each.

Temperature	-2.5°C		-3.0°C		-3.5°C	
Treatment / Genotype	Trifol	Unifol	Trifol	Unifol	Trifol	Unifol
P.ac 78	90*	54*	52	56	42	24
P.ac 19	50	28	20	58	12	34

* represents significant difference between Unifol and Trifol treatments (P<0.05).

4.5. Tolerance to sub-zero temperatures of parental and interspecific hybrids progeny based on electrolyte leakage.

An electrolyte leakage test was conducted on the different genotypes at similar temperatures to the ones used in the whole plant freezing tests. Based on the results of this type of freezing test, *P. acutifolius* W6 15578 was the most tolerant genotype: significantly better than *P. vulgaris* NY5-161 and ICA Pijao as well as *P. angustissimus* PI 535272 and *P. acutifolius* PI 430219 when exposed to -2.5°C and -3°C (all $P<0.05$; Fig. 4.5 left). It was not significantly different from the F_1-1 and BC_1-1 genotypes when evaluated at -3°C ($p=0.0011$).

Results for the F_1 were intermediate the two parents: significantly more tolerant than the NY5-161 parent at -3°C ($P=0.0011$; Fig. 4.5 left). The only BC_1 genotype that could be tested was similar to the F_1 in levels of tolerance at all temperatures tested and significantly better than NY5-161 when tested at -3°C ($P=0.0011$; Fig 4.5 left).

The levels of tolerance demonstrated by the BC_2 genotypes were variable. The BC_2 genotypes all had a similar percentage of electrolyte retention when exposed to -2.5°C; not significantly different from the parental controls (Fig. 4.5 right). BC_2 genotypes A and C had significantly better electrolyte retention than NY5-161 when exposed to -3°C ($p=0.0039$; Fig 4.5). When the temperature was decreased further, the differences between most of the BC_2 genotypes and NY5-161 were no longer significant, except for BC_2-F at -3.5°C ($p=0.02$; Fig 4.5 right). When the BC_2 were compared to each other, BC_2-A was significantly better that the rest when exposed to -3°C ($p= 0.01$), but at any other temperature there were no significant differences.

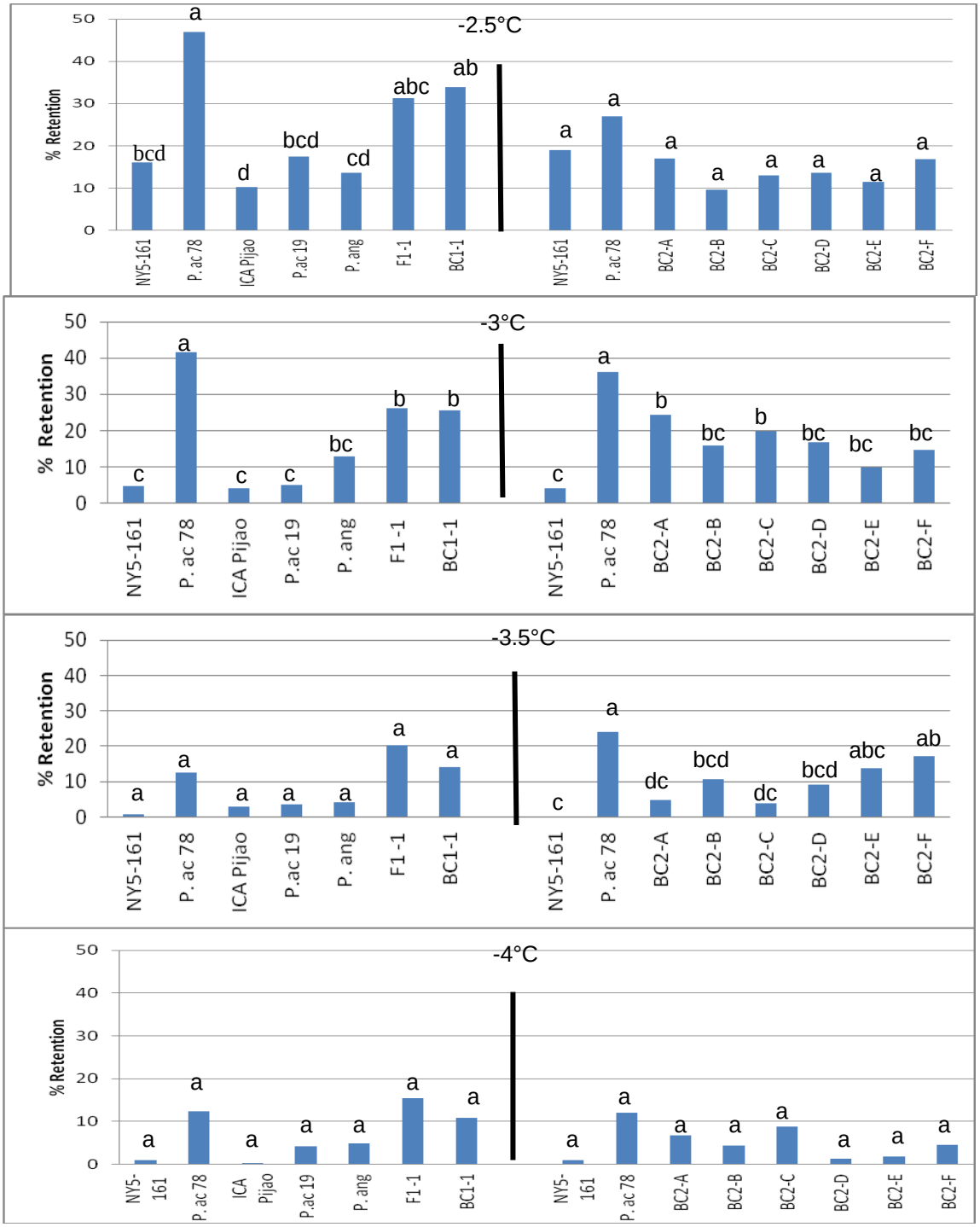

Figure 4.5. Electrolyte retention of *Phaseolus* genotypes ICA Pijao, NY5-161, W6 15578 (P. ac 78), PI 430219 (P. ac 19), *P. angustissimus* (P. ang), F_1-1 (NY5-161 X W6 15578), BC_1-1 (F_1 X NY5-161), and BC_2's A, B, C, D, E and F following exposure to -2.5°C, -3.0°C, -3.5 °C and -4.0°C, based on an electrolyte leakage test. Bars with the same letters are not significantly different from one another ($p>0.05$).

5. Discussion

When growing *Phaseolus* genotypes in the field in temperate regions, sometimes a small difference in the level of tolerance to sub-zero temperatures can represent the difference between harvesting some production out of a field, and losing everything.

Some accessions of *P. acutifolius* have been demonstrated to be a potential source of heat tolerance genes (Rainey and Griffiths, 2005). It also has been reported as having tolerance to drought (Shisanya, 2002). There have not been any studies that demonstrate *P. acutifolius* is a particularly cold tolerant species, but since it has some level of tolerance to both, heat and drought, two other abiotic stresses, it is possible that it also has some level of tolerance to exposure to cold temperatures. In a study done by Xiong et al., (2002), it was noted that drought, high salinity and cold are similar stress conditions that affect plants and that similar mechanisms are likely induced when plants are exposed to any of these conditions.

In order to assess the possibility of using *P. acutifolius* as a source of cold tolerance in *P. vulgaris*, a series of experiments was conducted. A preliminary test indicated that W6 15578 was a potential donor source so interspecific crossing and backcrossing was initiated while confirmation of the level of cold tolerance in this line was carried out. This resulted in a saving of time in the development of hybrids for further testing.

5.1 Hybrid Characterization

In order to transfer genes controlling traits from one species to another, interspecies hybridization must be possible. *Phaseolus acutifolius* accessions have been shown to hybridize well with *Phaseolus* genotypes (Thomas and Waines, 1984) and hybrids developed from this type of interspecific cross have also been used in a backcrossing program (e.g. Haghighi and Ascher, 1988). There is an incompatibility locus carried by some *P. vulgaris* genotypes that can make it difficult to carry out interspecific hybridization, but others, such as ICA Pijao and Sacramento Light Red Kidney, do not appear to carry this gene, thus allowing hybridization to occur (Parker and Michaels, 1986). NY5-161 was chosen for this crossing program because it is known to be more tolerant to cool temperatures (Dickson and Boettger, 1984) and is more adapted to SK growing environments. It had not been used in interspecies crosses before but apparently it too does not carry the incompatibility locus,

since the F_1 hybrids from crosses with *P. acutifolius* W6 15578 were successfully produced for this project using embryo rescue (Gurusamy, pers. comm.).

The morphological characteristics of the parents were clearly different (Table 4.4). For instance, NY5-161 had large white flowers with some light pink, while the W6 15578 accessions had small flowers with a strong pink color. The F_1 hybrids had medium sized flowers with an intermediate pink color, showing influence from both parents. Other characteristics were also intermediate, similar to the results that Mok et al., (1978) obtained when producing hybrids between *P. vulgaris* and *P. acutifolius.*

For the backcrossing, the cultivated parent, NY5-161, was used as the female. The morphological characteristics of the BC_1s was variable, some of them were closer to the *P. vulgaris* parent and some of them closer to the *P. acutifolius* parent. This demonstrated the segregation of genes that was occurring. The six BC_2 that were developed using embryo rescue showed characteristics even more similar to NY5-161. This was expected since the BC2 in theory has a bigger percentage of the genome from this recurrent parent.

The BC_2 plants were able to produce BC_3 seed without tissue culture intervention. In shape, the BC_3 seeds resembled the NY5-161 parent but there was variation in the seed coat color for the different genotypes (Fig 4.1). With the increase in percentage of the NY5-161 genome, the expectation was that many of the characteristics of this parental line would be present in the BC_2. This was desired since we were intentionally trying to recover the highest percentage of the cultivated genotype, but hopefully integrating some of the *P. acutifolius* genome, particularly regions carrying the genes that confer tolerance to exposure to sub-zero temperatures. Tolerance to sub-zero temperature exposure most probably is determined by the action of several genes present in different regions of the genome. Should some levels of tolerance have been transferred from the wild species into the hybrids then, with selection, some level of tolerance would remain in further generations. This would allow breeders to use this material to develop cultivated lines that will present some level of tolerance to low temperature exposure.

A pollen viability test was done on all the genotypes that were available. Using NY5-161 and W6 15578 as the standards, the pollen viability test showed clearly that the F_1 hybrid had the lowest percentage of fertility and how this increased with each generation of backcrossing. A similar result was obtain by Haghighi and Ascher, (1988) where they found that the percentage of pollen viability was lower in early generations but increase with further generation of backcrossing. This problem is likely due to the genomic imbalance that existed in the F_1 which decreased as the generations advanced and the genome became more like the

recurrent parent. Compared with the study done by Haghighi and Ascher, (1988), we were able to harvest seeds from the BC_2 plants, while they reported that they were only able to harvest seed from the plants in the BC_3 generation.

5.2 Tolerance to sub-zero temperatures in *P. acutifolius* genotypes

To test the theory that *P. acutifolius* is more tolerant to sub-zero temperatures than is *P. vulgaris*, a series of experiments was conducted. A set of accession are different from the one tested by Balasubramanian, (2004), were selected for initial testing. Whereas Balasubramanian selected *P. acutifolius* accessions from high altitude locations, assuming these would be more likely to be cold tolerant, the ones that were selected for this study originated in semi-desert areas and therefore would potentially have tolerance to drought.

Limitations of space in growth cabinets designed for experiments at sub-zero temperatures placed severe restrictions on the experimental design for testing the material. As a result, the first test on a larger set of lines was done with only four plants per line and was only conducted once. This was simply to narrow down the choice of lines for further study. The *P. acutifolius* lines were compared to the non-tolerant *P. vulgaris* line ICA Pijao and to the more tolerant *P. angustissimus* line reported by Balasubramanian et al., (2004) to see if any lines were as good as, or better than, the *P. angustissimus* line. The results showed promising levels of tolerance to sub-zero temperatures in some *P. acutifolius* accessions (Table 4.1), although data from only four plants means only very large differences can be detected. The two accessions from the subspecies *acutifolius* appeared to be more tolerant than the two from subspecies *tenuifolius*, which happens to be the same subspecies tested by Balasubramanian et al., (2004). In their study, the *tenuifolius* line (PI535248) tested had a level of tolerance to exposure to sub-zero temperatures that was between that of *P. vulgaris* and *P. angustissimus* PI535272, which is similar to the results found for the *tenuifolius* lines tested in this current experiment.

While accessions from both subspecies examined have their origin in Mexico, there were differences in survival and morphology. The leaf shape of *P. acutifolius* spp. *tenuifolius* is different with a more slim leaf type and a less succulent structure as compared to *P. acutifolius* spp. *acutifolius* which has a more robust leaf structure with bigger and more succulent leaves. These morphological characteristics may be contributing to the different levels of tolerance to low temperature stress that these two sub-species demonstrated. This is the first study that shows differences in the levels of tolerance to sub-zero temperature exposure among these sub-species.

Based on these initial results, the number of genotypes was reduced to four and the number of repetitions was increased to three to increase the ability to discriminate among the tolerant and non-tolerant *P. acutifolius* lines. The two *P. acutifolius* accessions chosen: W6 15578 and PI319445 were the ones that showed greater tolerance to exposure to sub-zero temperatures in the first experiment. The susceptible *P. vulgaris* cv. ICA Pijao and *P. angustissimus* PI535272 were also included in the follow-up experiment. The results from this experiment showed that *P. acutifolius* genotype W6 15578 was more tolerant to sub-zero temperatures than *P. vulgaris* cv. ICA Pijao.

In the final round of testing, it was confirmed that differences exist among the *Phaseolus* genotypes and that the *P. acutifolius* accession W6 15578 had the highest level of tolerance among all the genotypes tested. Taken together, these results confirm that W6 15578 is a potential source of genes that confer tolerance to stress due to exposure to sub-zero temperatures and its use in interspecific crosses with *P. vulgaris* should continue to be explored. Although this accession showed potential as a source of tolerance genes it would still be sensible to evaluate more accessions that exist to explore the possibility of finding a genotype that can provide even better levels of resistance.

It was only possible to test a few accessions due to availability of the germplasm and the limited space available. To test more germplasm a different methodology needs to be explored to be able to test the more plants more effectively.

5.3 A pre-freezing treatment as a trigger to increased tolerance to sub-zero temperature exposure

In some species, particularly cereals, exposure to low temperatures for a period of time confers some adaptation mechanism to allow plants to tolerate a lower temperature exposure than can un-treated plants. This phenomenon is known as acclimation (review by Thomashow, 1999). Mohapatra, (1987) found that alfalfa plants exposed to such a treatment did have increased levels of tolerance to sub-zero temperatures. Acclimated White clover (*Trifolium repens* L.) plants were reported to experience less dehydration than similar plants that were not acclimated (Guinchard et al., 1996). Balasubramanian, (2004) found that there was no significant difference in level of tolerance to sub-zero temperatures among *Phaseolus* genotypes that were exposed to the different period of exposure to chilling temperatures that he tested (7/5^{o}C, 5/2^{o}C and 2/0^{o}C). Based on his results, the decision to expose the plants to

an acclimation period of three day was taken. He did not test the different genotypes without any days of pre-freezing treatment.

An experiment was set up in order to test if exposure to a pre-freezing treatment would have an effect on the levels of tolerance to sub-zero temperature in *P. acutifolius* genotypes. The results showed that the exposure to low but non freezing temperatures for three days did not in general have any significant effect on the level of tolerance to exposure to sub-zero temperatures. The only exception was for W6 15578 when exposed to -3.5°C where the non pre-treated plants had a significantly higher percentage of survival. The results suggest that the acclimation process in this *Phaseolus* species not unlike other warm-season crops such as tomato, cotton, soybean and maize (Chinnusamy and Zhu, 2007). This dramatic drop in temperature is unlikely to occur in nature, however; and may actually be affecting the response although not in the traditional sense of acclimation. Further studies need to be done to determinate the actual effect in *Phaseolus* species.

5.4 Levels of tolerance at the unifoliate vs. third-trifoliate stage

A different methodology to evaluate the tolerance to sub-zero temperatures in different genotypes should be developed since space limitation in controlled growth chambers is a factor that reduces the capability for screening large numbers of accessions for cold tolerance. One possibility would be to screen at an earlier growth stage when individual plants are much smaller and therefore more plants can be tested per unit area.

It has been observed in tomato, another warm season crop with little tolerance to low temperatures, that the limited tolerance is independent of the growth stage (Damidaux and Martinez, 1992). This does not appear to have been tested in *Phaseolus*. Under Saskatchewan growing conditions, plants will usually be exposed to sub-zero temperatures under field conditions at an early growth stage, thus there is a clear need to develop lines that can support this kind of stress at an early growth stage, such as the unifoliate stage. This would also help address the problem of space restrictions as many more small plants can be screened in the same space than plants at the third-trifoliate stage.

Unfortunately, the W6 15578 genotype appeared to be slightly more susceptible at the unifoliate stage than the third-trifoliate stage. In contrast, PI430219 appeared to be more tolerant. These inconsistencies mean the unifoliate test is not a good predictor of the reaction at the third-trifoliate stage. The results, however, were not very convincing and the

experimental design, determined by cabinet space, made it difficult to truly assess the true differences.

Based on the results obtained in this experiment, evaluating genotypes at the unifoliate stage is not recommended for estimating tolerance at the trifoliate stage. Further assessments, however, need to be done to determine if there is any real difference when the plants are evaluated at a younger growth stage, since this stage is critical for the success of the crop on the Saskatchewan prairies.

5.5 Introgression of genes for tolerance to sub-zero temperatures into common bean

With the capability of exploring a large number of wild accessions of *Phaseolus* and the possibility of using interspecific crossing as a tool to introduce sub-zero stress tolerance genes into the cultivated bean, it may be possible to increase tolerance to sub-zero temperature exposure in *P. vulgaris.*

Since the F_1 hybrids and individual backcrosses that were developed were each unique due to the specific gene combination that occurs when they are formed and since it was our intention to know if the genes that influence the tolerance to low temperature stress was being transferred from one generation to another, a different methodology to evaluate tolerance needed to be identified. Even though the whole plant freezing test seemed to be effective at detecting differences among the genotypes, it is a destructive method that cannot be used for unique plants; i.e. those that cannot be replicated in large numbers. We were also unable to clone the hybrids in an efficient manner, so there was a need of an alternative to whole plant freezing that could allow us to evaluate tolerance levels in plants without need for a large number of identical plants.

5.5.1 Electrolyte leakage as an alternative method to evaluate levels of tolerance to exposure to sub-zero temperatures

The electrolyte leakage test is a non-destructive method used to screen for differences in freezing tolerance. This methodology was used by Nunes et al., (2003) to screen genotypes of the forage legume Rose clover (*Trifolium hirtum* All.), and by Cottee et al., (2006) to study cotton (*Gossypium hirsutum*). In the Cottee et al. (2006) study the results obtained in the electrolyte leakage test were comparable to the results obtained in the whole plant freezing test suggesting it is a reasonable substitute test. The main objective of this study in bean was to test this method for quantifying the different levels of resistance among different

genotypes, so selection among different genotypes, including interspecies hybrids, could be done in the future.

P. angustissimus PI535272, a species that was more tolerant to sub-zero temperatures using whole plant freezing, did not appear to be tolerant based on the electrolyte leakage test. It is possible that this species is not actually more tolerant but rather avoids freezing due to its morphology; the leaves from this species are smaller and thicker than the ones from the other species. The morphology of the leaf can suggest that the effect of the test on this particular genotype is different from the other ones due to the leaf structure.

The results from the other species tested were more comparable to the results obtained with whole plant testing. The genotype that was the most tolerant to exposure to sub-zero temperatures as whole plants also had a higher percentage of electrolyte retention. At the same time, the genotype that was the most susceptible to sub-zero temperatures as whole plants also had the lowest percentage of electrolyte retention. The results in the whole plant freezing test showed that the 50% mortality of *P. acutifolius* plants occurs when they are exposed to -3 ^{o}C, while in the electrolyte leakage test shows that when the samples are tested at -3 ^{o}C there is a 40% retention suggesting that this value might be use as a cut off for selecting individuals with increased capability to survive low temperature exposure.

A comparison of the results from the two different methodologies indicates that the electrolyte leakage test can be an effective initial method to evaluate the levels of tolerance to low temperature exposure stress. Tolerance should be confirmed using whole plant testing, however as the results are an indirect measurement of survival.

The F_1 hybrids seemed to have an intermediate level of tolerance to low temperature stress between the *P. vulgaris* parent and the *P. acutifolius* parent based on the electrolyte leakage results. This suggests that there is a contribution of genes to the level of tolerance of the hybrid from the wild species. The BC_1 plant evaluated was less tolerant the *P. acutifolius* parent, but it was still more tolerant than the cultivated species. This suggests that the genes that are affecting the levels of tolerance to low temperature stress are being retained in further generations than the F_1 hybrid.

The six BC_2 hybrids were also tested and generally had a better level of tolerance to low temperature stress that NY5-161 when subjected to the -3°C treatment. Only BC_2-F was significantly more tolerant when exposed to -3.5°C; although it was lower than the parent W6 15578, the F_1 hybrid and the BC_1 hybrid. This indicates that tolerance is continuing to be lost with further generations of backcrossing but that there are still some individuals that could be of interest.

In general the different methodologies used to screen for low temperature exposure tested here are useful for screening material but each has limitations. One of the biggest limitations with the whole plant freezing test is its destructive nature which clearly eliminates the possibility of its use in a breeding program where interspecific hybrids are being evaluated. The need for controlled environment chambers that are capable of reaching sub-zero temperatures reliably is also a problem with whole plant testing. The electrolyte leakage technique is limited in its ability to clearly show the levels of tolerance of the different materials. However, this non-destructive technique could be used to screen plants in early stages of a breeding program or when screening interspecific hybrids, to eliminate the least tolerant lines early on. Regardless, all genotypes that successfully survive these screening techniques should undergo field trials to confirm if the tolerance carries through to the field level.

6. Conclusions and Recommendations

Based on the results obtained from this series of experiments, it can be concluded that there are significantly different levels of tolerance to exposure to sub-zero temperatures within *Phaseolus* species with the ***P. acutifolius*** spp. acutifolius line W6 15578 being the most tolerant of those tested so far. The increased level of tolerance however is minimal (approx 1^{o}C), which seems small but may be the difference between losing a crop and not in a Saskatchewan spring. The sub-species *tenuifolius* is probably no more tolerant than *P. vulgaris* and as such is not a good source of cold tolerance genes.

One of the other objectives of this set of experiments was to identify a methodology to screen genotypes for tolerance to exposure to subzero temperatures. To avoid the uncontrolled nature of field testing, this can be done as whole plant tests in control environment chambers. Unfortunately this methodology is expensive, is severely limited by space, and it is also destructive which is a problem when working with unique genotypes like the ones developed through interspecific hybridization. Based on this, an alternative methodology needed to be identified, and the one tested was the electrolyte leakage test following exposure to sub-zero temperatures in a cryo-bath. Using this method it was possible to show differences in the levels of tolerance among the genotypes tested. This kind of test gave similar results to the whole plant freezing test with the genotypes that had the higher level of retention in the electrolyte leakage test being the ones that had better levels of tolerance in the whole plant freezing test. Preliminary screening to reduce the number of accessions to be evaluated in the chamber could, therefore, be done using the electrolyte leakage technique eliminating the ones that are obviously not tolerant low temperature exposure.

Finally, based on the results obtained using the electrolyte leakage technique, there appears to be some potential for increased levels of tolerance to sub-zero temperatures in some of the BC_2 hybrid plants over the susceptible, recurrent *P. vulgaris* parent NY5-161. This suggests it is worth continuing to test these plants and their progeny for tolerance to low temperature exposure; preferably as whole plants.

6.1 Recommendations

- More *P. acutifolius* accessions should be evaluated for tolerance to low temperature stress.
- Explore the option of testing indirectly for low temperature exposure such as evaluating other kind of stresses like drought tolerance or salt tolerance
- Cultivars and breeding lines that already have some *P. acutifolius* in their background should be evaluated as well for tolerance to low temperature stress.
- Field trials need to be done with the different genotypes to probe the levels of tolerance as well as with the breeding lines that are being developed.
- Initial screening of interspecific hybrids should be done only using electrolyte leakage to avoid any risk of losing the genotypes.
- Chambers studies can be limited to genotypes that have showed some levels of tolerance with the electrolyte leakage.
- More studies need to be done in order to evaluate the acclimation process in *Phaseolus spp*.
- Congruity backcrossing may be an alternative method to develop lines with tolerance to low temperature exposure stress. Since it has been prove that by using this technique there is and increment of introgression of genes as explain by Anderson et al., 1996.
- The used of large sample in the BC_2 generation can increase the chance of finding more lines with tolerance to low temperature stress.
- Tepary bean by itself is used in different regions for consumption. Since it carries different resistances it could be used as an alternative crop for regions where dry bean cannot be grow due to increased stress levels.

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