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Heritability of Cold Tolerance (Winter Hardiness) in *Gladiolus xgrandiflorus*

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1. Introduction

Gladiolus(-i) are herbaceous perennials with long, sword-like leaves and tall spikes of showy, colorful flowers (Goldblatt et al., 1998). Numerous cultivars (>10,000) have been bred (Sinha & Roy, 2002) with extended vase life, floral novelty, or extended flowering periods (Kumar et al., 1999; Takatsu et al., 2002). Recent focus has included transformation for potential creation of a genetically modified organism (GMO) cultivar (Kamo, 2008).

Most of the 180 *Gladiolus spp.* are originally from South Africa (Duncan, 1996, 2000; Goldblatt, 1996; Goldblatt & Manning, 1998; Goldblatt et al., 1993; Manning et al., 2002), although they are widely distributed to as far north as Russia and into the Mediterranean. In the Cape Province region alone, as many as 72 species have been identified as being native (Kidd, 1996). Winter-hardy species from Russia include *G. imbricatus* and *G. palustris.* It has been reported that several species are adaptable to cultivation, including *G. alatus, G. angustus, G. cardinalis, G. carmineus, G. carneus, G. dalenii, G. ochroleucus, G. pritzelii, G. saundersii,* and *G. sempervirens* (Duncan, 1982).

Most modern cultivated gladioli, *Gladiolus xgrandiflorus* Hort. (=*G. hortulanus*), are tetraploid (*2n=4x=60*) interspecific hybrids and have been cultivated for >260 years (Goldblatt, 1996). Modern *G. xgrandiflorus* hybrids are derived from n=6-12 S. African species (Barnard, 1972). Modern gladioli are primarily grown as summer-growing cut flowers and tender annuals. They are derived from summer-growing species, including *G. dalenii, G. oppositiflorus, G. papilio,* and *G. saundersii. Gladiolus cardinalis*, a winter growing species (winter rainfall region), has also been used in hybridization. The dwarf modern gladioli, 'Nanus' hybrids' (=*G. nanus*), are also interspecific hybrids derived from *G. tristis, G. carneus, G. primulinus,* and *G. cardinalis* (Goldblatt, 1996; Goldblatt & Manning, 1998). A new series of miniature gladiolus has also been derived from crossing the cultivated hybrids with wild species, i.e. [*G. xgrandiflorus*] *x G. tristis* (Cohen & Barzilay, 1991).

There are several environmental factors that affect the winter hardiness trait, including low temperatures, variable snow/ice cover, low light periods, and secondary invasion by pathogens (Blum, 1988; Tcacenco et al., 1989; Walker et al., 1995). Winter hardiness is a necessary trait for herbaceous perennials growing in northern climates and is important for floriculture crops as well as consumers (Kim & Anderson, 2006). Underground storage organs in geophytes, e.g., corms, bulbs, tubers, rhizomes, etc., allow herbaceous perennials to survive cold winters. The underground structure of perennial *Gladiolus* is a corm or fleshy storage stem from which shoots and roots grow. *Gladiolus* is a genus that has not been studied to any great extent in the area of winter hardiness. Bettaieb et al., (2007) found that low temperature stress of 8°C caused increased catalase (CAT) activity and lower hydrogen peroxide (H_2O_2) levels in gladiolus, but such information has not resulted in breeding for winter-hardy gladioli. Most or all cultivars are 'non-hardy' in Minnesota and other northern latitudes (Anderson, unpublished data).

The gladiolus breeding program at the University of Minnesota is part of a larger project in the Herbaceous Perennial Breeding Program to revolutionize geophytes. Gladiolus which are winter hardy or cold tolerant in USDA Zones 3-4 would allow this crop to overwinter in northern growing conditions and eliminate the need to dig the corms each fall and replant the subsequent spring. In recent years, due to the lack of adequate snow cover and cold temperatures the breeding program has had to supplement field overwintering over successive winters with laboratory freezing tests—a routine procedure widely used for woody and herbaceous perennial plants (Kim & Anderson, 2006). Our studies with USDA Z4 winter hardy chrysanthemums and gaura have shown that herbaceous perennial crowns and root systems must tolerate temperatures of –10°C (Z4) or –12°C (Z3) to survive Minnesota winters (Kim & Anderson, 2006; Pietsch et al., 2009). Most likely this is the case for gladiolus, although to the best of our knowledge, there have not been prior laboratory freezing tests of gladiolus corms for this purpose.

There are three inter-related research objectives for this study. First, selected cultivars and selections of *Gladiolus xgrandiflorus* will be tested to determine the range of cold tolerance at temperatures of 0°C to –10°C for all corm tissues and their subsequent regrowth potential. The second objective will be to determine the nuclear DNA genetic variation (using inter-simple sequence repeats, ISSRs) of the tested genotypes in comparison with wild species and other hybrids. Third, the heritability (h^2) of cold tolerance will be assessed in hybrids derived from crossing tested winter hardy and non-hardy parents.

2. Materials and methods

2.1 Experiment 1: initial freezing tests to determine cold tolerance response

Six cultivars or numbered breeding selections of *G. xgrandiflorus* were used in this study: 'Gemini', 'Great Lakes', 'King's Gold', 'Lady Lucille', Sel'n. 95-49 and 98-29 (Don Selinger, Minnesota Gladiolus Society, Woodbury, MN, USA). Prior to receipt, plants were field-grown in 2005 (45°N lat.) under a natural progression of short days prior to June (June 1=15.2 h), long days in mid-summer, to short days in September (Sep. 1=13.25 h; United States Nautical Almanac Office, 1977); the soil type was a Waukegan silt loam. Mean temperatures (monthly highs, lows) during the 2005 growing season (May 1 – Sep. 30) ranged from 24.5±8.1/10.9±3.8°C (day/night) (University of Minnesota, Department of Soil,

Water & Climate, 2005). Mature corms from plants which had flowered were dug in weeks 40-41 (2005), dried down (~21°C) for 2-3 weeks and shipped. Upon receipt, corms were cooled at 2°C in darkness (Widmer, 1958) for >1,000 h. In week 2 (2006), mature corms were planted into 11.4 cm^2 square deep PF Landmark containers (Belden Plastics, St. Paul, MN, USA) in trays filled with Sunshine #8/LC8 Professional Growing Mix (SunGro Horticulture, Bellevue, WA USA). Potted corms returned to 2°C (dark) until week 4 (2006) when the programmed freezing tests began.

2.1.1 Programmed freezing tests

There were 5 reps/treatment (corms) x 4 treatments (freezing temperatures) for a total of 20 experimental units for each genotype included in the programmed laboratory freezing tests (Kim & Anderson, 2006). Freezing tests were conducted using a Tenney environmental growth chamber (Model No. T20S, Series 942; Tenney Environmental Lunaire Co., Williamsport, PA, USA) with a programmable Series 942 Ramping Controller (Watlow Controls, Winona, MN). This created precision in the "profile control" of pre-programmed multi-step ramp (linear temperature change) and soak (constant temperature) times (Waldron, 1997). Precision temperature data loggers (Veriteq SP-1000; Veriteq Instruments, Inc., Richmond, British Columbia, Canada) were buried in the center (adjacent to the planted corms) of randomly selected pots throughout the chamber to ensure the ramp and soak temperatures were actually obtained. Once the pots were placed in the growth chamber (+2°C) in a randomized complete block (RCB) design, 15.25 h elapsed after transfer for the soilless medium to return to +2°C. Cold tolerance was assessed at 0°C, -3°C, -6°C, and -10°C with 5 h ramp time periods (5 h each for +2°C to 0°C, 0°C to -3°C, -3°C to -6°C, -6°C to -10°C) and a 13.25 h soak time at each temperature treatment (0°C, -3°C, -6°C, -10°C) following completion of each ramp down period (Kim & Anderson, 2006). After each treatment, samples were removed for cold damage assessment upon completion of each soak time and placed into a cooling chamber at 2°C for 2-3 d (dark) until the soilless medium was completely thawed (>0°C).

2.1.2 Regrowth assay

Once thawed, potted corms for each temperature treatment were moved to glasshouse conditions of 20/17 °C (day/night) temperature with a 16 h photoperiod (0800-2000 HR) to assay freezing damage to corms and regrowth potential of roots from the basal plate and shoots from the apical meristem. Three weeks later, plant and corms samples were harvested; soilless medium was washed away from the roots. Root number, root lengths (cm), shoot number, and shoot lengths (cm) were recorded. Root and basal plate cell damage (live=white, dead=brown to necrotic) was also determined. Corm damage was assessed after cutting each corm exactly in half with a vertical cut. Tissues were scored on a 1-5 scale, with 1=dead (completely brown or black), 2=brown coloration, 3=green or yellow coloration, 4=slight discoloration, and 5=completely undamaged (Fuller & Eagles, 1978; Perry & Herrick, 1996; Kim & Anderson, 2006; Waldron, 1997).

2.1.3 Data analyses

The determination of LT_{50} (the temperature at which 50% of the population sample is killed) was calculated for each genotype (Pomeroy & Fowler, 1973; Tcacenco et al., 1989).

Quantitative data were analyzed by Analysis of Variance (ANOVA) and mean separations using Tukey's Honestly Significant Difference (HSD) test at α=0.05 using the Statistical Package for the Social Sciences (SPSS), Ver. 9.0 (SPSS, Chicago, IL, USA).

2.2 Experiment 2: molecular analysis of potential parents

Twenty genotypes of *Gladiolus species* (wild species accessions, hybrid cultivars, and numbered selections), including those used in Experiment 1, were used in this study. These represented a diversity of non-winter-hardy and winter-hardy accessions pre-screened (2005-2008) by the University of Minnesota Gladiolus Breeding Program: ten wild *Gladiolus spp.* (*G. callianthus* [Code: Acidanthera], *G. byzantinus*, *G. carinatus* [1TRB, 2TRB, 3TRB], *G. dalenii* [GL-DAHL], *G. geardii* [GL-Gear], *G. gracilis* [GL-GRAC], *G. saundersii* [03 Gsaun-SS 03-61], *G. tristis* var. *concolor* [GL-TRIS]), five named cultivars (*G. nanus* 'Impressive', *G. xgrandiflorus* 'Gemini', 'Great Lakes', 'King's Gold', 'Lady Lucille'), and five numbered selections (*G. xgrandiflorus* 95-49, 98-29, VT03, 04GL-27-6, 04GL-57-7). Seeds of wild species were obtained from Silverhill Seeds (Capetown, South Africa), numbered selections from Don Selinger (Sel'ns. 95-49, 98-29; Woodbury, MN, USA) and the University of Minnesota Gladiolus Breeding Program (VT03, 04GL-27-6, 04GL-57-7), while named cultivars were sourced from Don Selinger (Woodbury, MN, USA) and Holland Bulb Farms (Milwaukee, WI, USA). DNA was extracted from young leaf tissue of plants grown in a glasshouse (20/17 °C day/night, 16 h photoperiod 0800-2000 HR) using a cetyltrimethyl ammonium bromide (CTAB) protocol based on Doyle & Doyle (1987).

2.2.1 Polymerase Chain Reaction (PCR) conditions

Six UBC primers (University of British Columbia Vancouver, Canada, http://www.biotech.ubc.ca/frameset.html) with a high level of polymorphism and scorability (UBC 808, 810, 811, 813, 814, 818)—which have been successfully used with another monocot and geophyte (*Lilium longiflorum;* Anderson et al., 2010) and modifications of Roy et al. (2006) were used for PCR amplification and ISSR analysis. Each PCR contained 0.25 units Flexitaq™ DNA polymerase, 20µM of a single primer, 10mM dNTP, 1.0mM $MgCl_2$, 2µL diluted DNA solution and 5x Flexibuffer™, which was supplied with the polymerase, for a total volume of 25 µL in each reaction (Yamagishi et al., 2002). Amplifications were carried out in a thermocycler (PTC-100, MJ research Inc., Hayward, CA 94545, U.S.A.). Amplification conditions were 7 minutes of denaturation at 94°C followed by 50 cycles of temperatures [94°C for 30 seconds, 43°C for 70 seconds, 72°C for 120 seconds and a final 7 minute extension at 72°C]. Each sample was replicated thrice.

2.2.2 Gel electrophoresis

A 1.5 % agarose (mixed with 100 ml of electrophoresis, 1XTris Acetate) buffer was made and heated in a microwave at high power for ~2 mins. until completely melted. Ethidium bromide was added to the gel (final concentration 0.5 ug/ml) at this point to facilitate visualization of DNA after electrophoresis. Electrophoresis chambers were filled with 1XTris Acetate EDTA plus 5 µg/ml ethidium bromide (Sambrook et al., 1989). Sample DNA volumes (12 ml) were then loaded and a current of 75 Volts for 2~2.5 hours was applied. Gels were visualised under UV light and recorded using a Fluro Chem 500 camera (Alpha Innotech Corp., San Leandro, CA, USA).

2.2.3 Data analyses

Unequivocally scorable and consistently reproducible amplified DNA fragments for the primers (UBC 808, 810, 811, 813, 814, 818; Anderson, et al., 2010) across all three replications were transformed into binary character matrices (1 = presence, 0 = absence). Cross Checker was used to score and analyze binary interpretation of DNA fingerprints (http://www.spg.wau.nl/pv/pub/CrossCheck/). Variation was measured using Hedrick's Index (*I*) (Hancock & Bringhurst, 1979). To obtain interpopulation variance estimates, Russell & Rao (1940) coefficients of similarity were calculated. Clustering, dendrograms were created by the unweighted pair group method (UPGMA) using the SAHN-clustering and TREE programs from NTSys version 2.02 (Computer Exeter Software, Setauket, NY, USA).

2.3 Experiment 3: heritability of cold hardiness

In this experiment laboratory freezing tests were used to determine the level of heritability (h^2) (Kim & Anderson, 2006). Three population groups of segregating interspecific F_1 hybrids, derived from crossing *G. xgrandiflorus* hardy (H) or non-hardy (NH) parents (H x H, H x NH, NH x NH), were analyzed for cold hardiness. Since most of the F_1 hybrids are derived from interspecific hybrid parents, the F_1 generation is most likely to segregate (rather than the F_2; Anderson & Ascher, 1996). Seeds from 122 crosses or self pollinations (8,833 seeds) between the *G. xgrandiflorus* genotypes tested in Experiment 1 and an additional hardy numbered selection (VT03) or its inbred seedlings (VT03-2, -3, -5) made in 2006 were germinated. Due to low germination rates and seedling numbers, a total of 375 genotypes/population group were analyzed (Table 1).

The F_1 hybrids were grown in the glasshouse in 7.6 cm^2 containers filled with pasteurized gladiolus soilless mix (40% peat moss, 60% sand) under long day photoperiods (0600-2200 HR light) at 19/17°C (day/night) temperatures for 6 wk., followed by a dry down period of 4 wk at 21°C and darkness (to mimic summer dormancy). At the end of the dry down period, the corms were repotted in the gladiolus soilless mix and acclimated at 2°C for 1 wk prior to freezing tests.

A total of 375 corms/group were selected for the study (Table 1). The experimental design was a completely randomized design (CRD), consisting of 5 temperatures x 3 population groups x 5 freezing runs x 15 corms/population group/temperature, for a total of 1125 experimental units (corms). The five temperatures tested (0°C, -3°C, -6°C, –10°C, -12°C) included the four from Experiment 1 with an additional lower temperature (-12°C) in case the H x H genotypes were heterotic and tolerated lower temperatures than their parents. Corms from each of these three population groups were moved to the Tenney environmental growth chamber and re-acclimated at 2°C for 15.25 hours (*cf.* Experiment 1). Since the containers were smaller than those used in Experiment 1, the ramp times were shortened to 5 hr with a 2 hr soak time at each test temperature. Otherwise, the experiment protocols for laboratory freezing, thawing, reforcing in the glasshouse, as well as data collection, were the same as Experiment 1. Broad sense heritability (h^2) estimates and confidence intervals were also calculated (Knapp et al., 1985; Fehr, 1987). These h^2 estimates are a ratio of the total genotypic variance (additive, dominance, and epistasis) to the phenotypic variance.

Population Group	Cross No.	Female Parent	x	Male Parent	No. Seedlings tested
H x H	06GL-62	98-29	x	VT03	133
	06GL-63	98-29	x	VT03-5	14
	06GL-65	VT03-2	x	VT03-3	50
	06GL-66	VT03-3	x	VT03-2	14
	06GL-78	Lady Lucille	x	95-49	50
	06GL-84	VT03	x	VT03-3	60
	06GL-117	98-29	x	Self	54
H x NH	06GL-29	04GL-57-16	x	98-29	35
	06GL-31	04GL-57-23	x	VT03-5	38
	06GL-32	04GL-57-24	x	98-29	66
	06GL-35	04GL-57-5	x	VT03	69
	06GL-61	98-29	x	95-49	89
	06GL-72	Great Lakes	x	VT03-5	78
NH x NH	06GL-37	04GL-57-7	x	Great Lakes	33
	06GL-73	King's Gold	x	95-49	61
	06GL-93	04GL-27-14	x	Self	52
	06GL-99	04GL-27-8	x	Self	49
	06GL-102	04GL-57-12	x	Self	38
	06GL-103	04GL-57-12	x	Self	41
	06GL-107	04GL-57-23	x	Self	5
	06GL-111	04GL-57-5	x	Self	96

Table 1. Population group (hardy x hardy [H x H], hardy x non-hardy [H x NH], non-hardy x non-hardy [NX x NH]), cross number, female/male parents of F_1 hybrid or selfed gladiolus crosses and the number of seedlings tested in Experiment 3 for heritability of winter hardiness. There were 375 seedlings tested within each population group for a total of 1,123 genotypes.

3. Results and discussion

3.1 Experiment 1: initial freezing tests to determine cold tolerance response

Mean root numbers ranged from 0.0 (King's Gold at 0, -3, -6, -10°C; 'Lady Lucille', 'Gemini', 'Great Lakes', Sel'n. 95-49 at -10°C; Sel'n. 98-29 at -6, -10°C) to 29.6 ('Great Lakes', 0°C) (Table 2). In the ANOVA, both genotypes and freezing temperatures as well as their interaction were very highly significant ($p \leq 0.001$) for root number and data could not be pooled. 'King's Gold' had no roots after any of the freezing temperature treatments, including 0°C, indicating that all root initials were killed (Table 2). In most instances, dead root initials occurred when the basal plate was completely dead (black). The significantly greatest mean number of roots occurred with 'Lady Lucille', 'Great Lakes' at 0, -3°C; Sel'n. 98-29 at -3°C (Table 2).

'King's Gold' had no living apical meristem (shoot) or root initial tissues with blackened basal plates (Fig. 1A). Mean root lengths showed similar responses to mean root numbers,

ranging from 0.0 cm long (King's Gold at 0, -3, -6, -10°C; 'Lady Lucille', 'Gemini', 'Great Lakes', Sel'n. 95-49 at -10°C; Sel'n. 98-29 at -6, -10°C) to 6.4 cm (Sel'n. 98-29, Table 2). If roots existed, most genotypes and freezing temperatures averaged ~1.0 cm root lengths with only 'Lady Lucille', 'Great Lakes' and Sel'n. 98-29 (-3°C) having the significantly longest roots (Table 2). Oddly enough these genotypes, which also had significantly higher root numbers at 0°C, had significantly shorter roots than at -3°C (Table 2). Both genotypes and freezing temperatures, as well as their interaction, were very highly significant ($p \leq 0.001$) for root number and data could not be pooled.

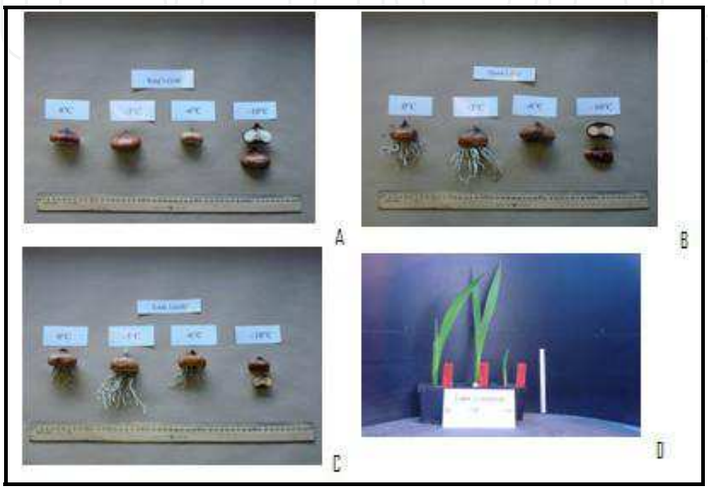

Fig. 1. Corm damage scoring examples of phenotypic responses in gladiolus cultivars and selections after programmed laboratory freezing tests at 0°C, -3°C, -6°C, and -10°C: 'King's Gold' (A), 'Great Lakes' (B), 'Lady Lucille' (C). Regrowth of shoots at 0°C, -3°C, and -6°C (D) for 'Lady Lucille' (the -10°C tretment was completely dead, *cf*. Fig. 1C).

The range in response among genotypes for the corm damage scoring (Fig. 1) is demonstrated by 'King's Gold' (Fig. 1A) with all root and shoot tissues dead, to 'Great Lakes' with living root/shoot tissues at 0, -3°C only (Fig. 1B), and finally 'Lady Lucille' with living root (0 to -6°C) and apical meristem (-3°C) tissues (Fig. 1C). Slight variations between replications occurred (Table 2). Corm damage values did not differ significantly for genotypes ($p=0.132$) or the interaction of genotype x freezing temperature ($p=0.272$), although freezing temperatures were highly significantly different ($p \leq 0.001$). Thus, genotypes could be pooled within temperature treatments (Table 2). The most severely damaged corms (1.0) occurred at -10°C which differed significantly from -3°C (4.23) (Table 2). Corm damage scores at 0, -3°C did not differ from each other and were the best and healthiest corms possible (nearly 5.0 on the 1-5 scale; Table 2). Since stem storage tissue in the corms was the primary score for corm damage, stem tissue is quite resilient to freezing (0, -3, -6°C all had significantly higher scores than -10°C; Figs. 1A, 1B, 1C, Table 2) although not enough to confer winter survival in USDA Z3-4 (Kim & Anderson, 2006; Pietsch et al., 2009). Significantly increased cold tolerance would be required to survive winter conditions in northern latitudes.

Most mean shoot lengths were at 0.0 cm, three weeks after the freezing tests, due to complete death of the apical meristem (Table 2). The significantly longest shoots occurred at the -3°C freezing temperature for 'Great Lakes' (0.84 cm) and Sel'n. 98-29 (1.74 cm) (Table 2). Example

shoot regrowth (Fig. 1D) later demonstrated that some stems had floral meristems and would flower (Fig. 1D, 0°C and -3°C) while others were thinner-stemmed and vegetative (Fig. 1D, -6°C). Genotypes were significantly different (p=0.026) whereas both freezing temperature treatments and genotype x freezing temperature interaction were highly significant (p≤0.001).

Genotype (cultivar, Sel'n.)	Temp. Trmt.	Root No.	Root length (cm)	Corm damage	Shoot Length (cm)
King's Gold	0°C	0.0±0.0a	0.0±0.0a	**4.8±0.4c**	0.0±0.0a
	-3°C	0.0±0.0a	0.0±0.0a	**4.97±0.0c**	0.0±0.0a
	-6°C	0.0±0.0a	0.0±0.0a	**4.23±0.0b**	0.0±0.0a
	-10°C	0.0±0.0a	0.0±0.0a	**1.0±0.0a**	0.0±0.0a
Lady Lucille	0°C	25.0±10.5c	1.1±0.8ab		0.0±0.0a
	-3°C	23.2±16.4c	2.5±2.3bc		0.3±0.5ab
	-6°C	16.6±10.8b	1.4±1.0b		0.0±0.0a
	-10°C	0.0±0.0a	0.0±0.0a		0.0±0.0a
Gemini	0°C	16.6±12.3b	1.0±0.8ab		0.0±0.0a
	-3°C	13.6±13.1b	1.4±1.8b		0.3±0.7ab
	-6°C	4.4±6.4a	0.75±1.3ab		0.0±0.0a
	-10°C	0.0±0.0a	0.0±0.0a		0.0±0.0a
Great Lakes	0°C	20.4±5.2c	1.8±0.7b		0.0±0.0a
	-3°C	29.6±5.8c	4.1±1.5c		0.8±1.0c
	-6°C	4.4±9.8a	0.3±0.6a		0.0±0.0a
	-10°C	0.0±0.0a	0.0±0.0a		0.0±0.0a
Sel'n. 98-29	0°C	9.1±7.8a	1.3±1.2b		0.0±0.0a
	-3°C	20.0±6.9c	6.4±3.7c		1.7±1.2c
	-6°C	0.0±0.0a	0.0±0.0a		0.0±0.0a
	-10°C	0.0±0.0a	0.0±0.0a		0.0±0.0a
Sel'n. 95-49	0°C	9.4±8.1a	1.7±2.6b		0.3±0.7ab
	-3°C	12.5±8.7b	1.8±1.4b		0.4±0.7b
	-6°C	1.0±2.2a	0.5±1.1ab		0.0±0.0a
	-10°C	0.0±0.0a	0.0±0.0a		0.0±0.0a

Table 2. Mean ±S.D. number of roots, root length (cm), corm damage (1-5 scale, with 1=dead (completely brown or black), 2=brown coloration, 3=green or yellow coloration, 4=slight discoloration, and 5=completely undamaged (Fuller & Eagles, 1978; Perry & Herrick, 1996; Kim & Anderson, 2006; Waldron, 1997) and shoot length of six gladiolus tested for across four freezing temperatures (0°C, -3°C, -6°C, -10°C). Pooled values (corm damage) are shown in **bold typeface.** Mean separations (5% H.S.D.) were performed post-ANOVA.

Lethal temperature (LT_{50}) values was -10°C for corm damage (stem tissue) with the exception of Sel'n. 95-49 (Table 3). Root number and lengths had LT_{50} values of -6°C for all tested genotypes with the exception of 'Lady Lucille' (LT_{50} = -10°C) and 'King's Gold' (LT_{50} >0°C) (Table 3). Shoot apical meristem LT_{50}s (shoot length measurements) were significantly higher at -3°C for 'Great Lakes' and >0°C for all other genotypes (Table 3). Overall, 'King's Gold' had significantly shorter shoots or fewer living apical meristems than Sel'n. 98-29

(which was the best). These LT_{50}s are similar in response to cold tolerant (LT_{50}=-10°C) to non-cold-tolerant (LT_{50}≤-6°C) garden chrysanthemum and gaura rhizomes (Kim and Anderson, 2006; Pietsch et al., 2009). Since there were far fewer treatment and genotype combinations with shoots, compared to roots, apical meristems are more sensitive to cold and freezing than root initials. Thus, for the six tested gladiolus genotypes, the apical meristem (shoots) is more sensitive to freezing than roots which are likewise more sensitive than stem tissue (corms) to freezing temperatures. One could expect corms to remain in the ground after a freeze/thaw cycle until they have respired to death and/or been invaded by pathogens.

Genotype tested (cultivar, sel'n.)	Root No.	Root length (cm)	Corm damage	Shoots (length; cm)
King's Gold	>0°C	>0°C	-10°C	>0°C
Lady Lucille	-10°C	-10°C	-10°C	>0°C
Gemini	-6°C	-6°C	-10°C	>0°C
Great Lakes	-6°C	-6°C	-10°C	-3°C
Sel'n. 98-29	-6°C	-6°C	-10°C	>0°C
Sel'n. 95-49	-6°C	-6°C	-6°C	>0°C

Table 3. Lethal temperatures for root number, root lengths (cm), corm damage, and shoot lengths (cm) at which 50% (LT_{50}) of the population of gladiolus corm tissues for six genotypes were killed in the chamber freezing tests.

3.2 Experiment 2: molecular analysis of potential parents

Previous researchers only used two primers to determine clonal fidelity in gladiolus (Roy et al., 2006). Four (UBC 808, 810, 811, 818) out of the six primers tested produced scorable, unequivocal bands, giving increased stringency. In the present study, 62 well-defined (clear and unequivocal bands) and scorable markers across replications were obtained. A previous gladiolus ISSR study (Roy, et al., 2006) had its own synthesized primers, rather than those readily available from the University of British Columbia (UBC). Several of these UBC primers were previously used in our laboratory to test genetic variation in clonal *Lilium longiflorum* (Anderson et al., 2010). A total of 38 (61.29%) primer pairs were polymorphic. Replications did not differ significantly in their banding patterns. Total numbers of scorable loci/primer ranged from 16 (Primer pair 808) to 19 (Primer pair 811) for all tested gladioli whereas the number of scorable, polymorphic loci/primer ranged from 8 (810, 818) to 12 (808).

The genetic variation for the tested genotypes (Experiment 1) range across a wide spectrum of the gladiolus genome (Fig. 2). A constructed dendrogram for the tested gladiolus nuclear DNA genetic variation delineates two principle groups separating at Nei's (1972) genetic distance (GD) of 0.53 (Fig. 2) with only one subsequent monophyletic singleton (a set with exactly one genotype) found ('Great Lakes', GD=0.638). Sel'n. 95-49 forms a distinct group at GD=0.74 with 'Lady Lucille' and 'Gemini' (Fig. 2); the latter two have a GD=0.718. Each principle group further separated into additional overlapping, contiguous clades (contigs) (Fig. 2). The remaining genotypes tested for freezing tolerance (Sel'n. 98-29, 'King's Gold'; Experiment 1) were distributed within a clade at GD=0.75 (Fig. 2). Several pairs of genotypes

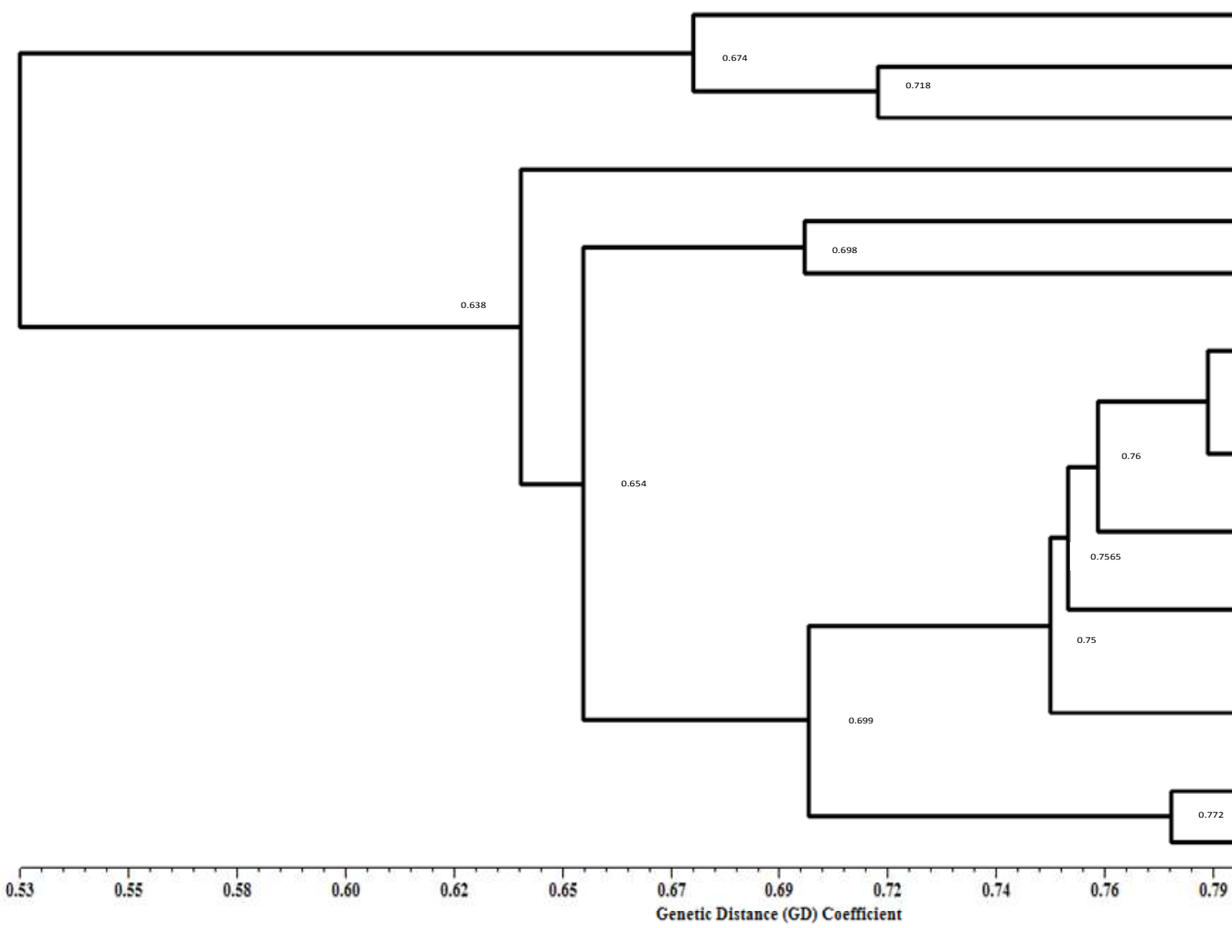

Fig. 2. Dendrograms of relatedness for wild *Gladiolus spp.* (*G. callianthus* [Code: Acidanthera], *G. byz*
2TRB, 3TRB], *G. dalenii* [GL-DAHL], *G. geardii* [GL-Gear], *G. gracilis* [GL-GRAC], *G. saundersii* [03 Gs
concolor [GL-TRIS]), five named cultivars (*G. nanus* 'Impressive', *G. xgrandiflorus* 'Gemini', 'Great La
Lucille'), and five numbered selections (*G. xgrandiflorus* 95-49, 98-29, VT03, 04GL-27-6, 04GL-57-7) d
scorable bands for five ISSR primers (UBC 808, 810, 811, 814, 818) and based on UPGMA analysis us

were very closely related and separated at GD=0.86, e.g., *G. gracilis* and *G. carinatus* 2 TRB; 'Impressive' and *G. byzantinus;* Sel'n. VT-03 and *G. dalenii; G. geardii* and *G. carinatus* 1TRB. Others separated at a lower level of genetic relatedness, i.e. Sel'n. 98-29 and 04GL-57-7 (GD=0.808), etc. Interestingly, Sel'n. VT-03 which has survived for >5 years in an unreplicated trial in USDA Z3 is closely related (GD=0.86) to *G. dahlenii,* introduced by Thornburn in 1908 (http://www.oldhousegardens.com/display.aspx?prod=SGL08), a source of cold hardiness.

3.3 Experiment 3: heritability of cold hardiness

Crossing groups, freezing temperatures, and their interaction were all highly significant ($p \leq 0.001$). In all crossing groups for -10°C, -12°C freezing temperatures, no living roots, root initials, apical meristems (shoots, shoot lengths) occurred (Fig. 3) and mean corm ratings for these temperatures (ranging from 1.1 to 1.2) barely exceeded the completely dead rating of 1.0 (Table 4). Thus, no transgressive segregants for corm ratings occurred with greater hardiness than the parents (Experiment 1, Table 2). In general, crossing groups involving at least one hardy parent (hardy x hardy, non-hardy x hardy) had significantly greater numbers and lengths of living roots and shoots than the non-hardy x non-hardy group.

Mean number of roots ranged from 0.0 (all three crossing groups at -10°C and -12°C) to 4.9 (non-hardy x hardy, -3°C) (Table 4). The significantly greatest number of roots occurred in hardy x hardy crosses at 0°C, -3°C (Fig. 3A) and non-hardy x hardy at -6°C (Fig. 3B). In all crossing groups and freezing temperatures, the number of roots was significantly lower than that found for the parents (Table 2). Average root lengths varied from 0.0 (all three crossing groups at -10°C and -12°C) to 6.89 (non-hardy x hardy, -3°C; Table 4) with the significantly greatest root lengths found in hardy x hardy at -3°C and non-hardy x hardy at -3°C, -6°C (Fig. 3). Root lengths, in some cases (6.89 for non-hardy x hardy at -3°C, Table 4), exceeded parental values (6.36 for Sel'n. 98-29 at -3°C, Table 2). Overall, roots in the progeny averaged longer lengths than the parents. Root number heritability ranged from $h^2 = 0.08$ (hardy x hardy) to $h^2=0.67$ (non-hardy x non-hardy) (Table 4). Root length heritability was both negative ($h^2=-0.14$, non-hardy x non-hardy crosses) to positive ($h^2=0.37$) (Table 4).

Corm ratings of the hybrids ranged from 1.1 (hardy x hardy and non-hardy x non-hardy at -12°C; non-hardy x hardy at -10°C) to 2.9 (hardy x hardy, 0°C) (Table 4). Heritability of corm ratings was low, ranging from $h^2=-0.04$ (hardy x hardy crosses) to $h^2=0.15$ (non-hardy x non-hardy) (Table 4). All corm ratings in all three crossing groups were significantly higher for freezing temperature treatmets of 0°C to -6°C than for lower temperatures (-10°C and -12°C) (Table 4). The parental values for 0°C to -6°C corm ratings (Table 2) were significantly higher than any of the progeny. These differences could be attributed to the significantly smaller corm size and stem tissue volume/density since the hybrids were only two-year-old corms (Fig. 3) and ~1/5 the size of the tested parental corms of commercial size (Fig. 1).

Fig. 3. Example freezing responses at 0°C, -3°C, -6°C, -10°C, and -12°C for gladiolus seedlings derived from A) Hardy x Hardy (HH), B) Hardy x Non-hardy (HN), and C) Non-hardy x Non-hardy (NN) parental crosses.

The average number of shoots among progenies ranged from 0.0 (all three crossing groups at -10°C and -12°C) to 1.1 (hardy x hardy at -6°C; non-hardy x hardy at 0°C, -3°C) (Table 4). In this case, shoot numbers >1.0 indicated transgressive segregation over the parents which all had 1.0 shoots/corm (Experiment 1). In all cases, a significantly greater number of shoots were found at the 0°C, -3°C, and -6°C temperatures for all crossing groups. Mean shoot lengths ranged from 0.0 (all three crossing groups at -10°C and -12°C) to 10.7 cm (non-hardy x hardy at -6°C, Table 4). Non-hardy x non-hardy crosses for 0°C, -3°C, and -6°C had significatly longer shoots than those with 0.0 cm lengths. Shoot lengths at 0°C, -3°C, and -6°C for both hardy x hardy and non-hardy x hardy groups were significantly longer than any other freezing temperatures within these groups or freezing temperatures for all non-hardy x non-hardy crosses (Table 4). When shoot lengths exceeded 0.0 cm for all crosses at 0°C, -3°C, and -6°C (Table 2), these were significantly longer than any parents (Table 2), indicating transgressive segregation for this trait. Heritability of shoot lengths remained low, however, ranging from h^2=-0.43 (hardy x hardy) to h^2=0.19 (non-hardy x hardy) (Table 4).

Crossing group	Temp. Trmt.	Root No.	Root length (cm)	Corm damage	Shoot No.	Shoot Length (cm)	No. (%) plants survived
Hardy x Hardy	0°C	4.0±2.9cd	5.7±4.8c	2.9±1.7b	1.0±0.2b	6.4±6.6c	17 (22.7%)
	-3°C	4.0±2.4cd	6.3±4.1d	2.7±1.8b	1.1±0.3b	6.7±5.3c	20 (26.7%)
	-6°C	3.4±2.0c	5.9±4.4c	2.7±1.7b	1.1±0.4b	8.5±7.6d	17 (22.7%)
	-10°C	0.0±0.0a	0.0±0.0a	1.2±0.5a	0.0±0.0a	0.0±0.0a	0 (0.0%
	-12°C	0.0±0.0a	0.0±0.0a	1.1±0.4a	0.0±0.0a	0.0±0.0a	0 (0.0%)
h^2		0.08	0.29	-0.04	---	-0.43	---
Non-hardy x Hardy	0°C	3.2±1.4bc	4.4±2.9c	2.3±1.6ab	1.1±0.3b	7.4±5.8c	9 (12.0%)
	-3°C	4.9±4.4cd	6.9±4.3d	2.7±1.8b	1.1±0.3b	7.5±9.5c	16 (21.3%)
	-6°C	3.5±1.7c	6.3±6.8d	2.0±1.5ab	1.0±0.0b	10.7±8.3e	8 (10.7%))
	-10°C	0.0±0.0a	0.0±0.0a	1.1±0.4a	0.0±0.0a	0.0±0.0a	0 (0.0%
	-12°C	0.0±0.0a	0.0±0.0a	1.2±0.5a	0.0±0.0a	0.0±0.0a	0 (0.0%)
h^2		0.31	0.37	0.08	---	0.19	---
Non-hardy x Non-hardy	0°C	2.1±1.5b	2.6±2.5b	2.3±1.6ab	1.0±0.0b	1.9±1.8ab	4 (5.3%)
	-3°C	2.8±1.6c	5.7±4.4c	2.0±1.5ab	1.0±0.0b	2.3±1.8b	4 (5.3%)
	-6°C	2.2±1.7b	2.5±2.2b	2.3±1.5ab	1.0±0.0b	2.6±1.8b	7 (9.3%)
	-10°C	0.0±0.0a	0.0±0.0a	1.2±0.5a	0.0±0.0a	0.0±0.0a	0 (0.0%
	-12°C	0.0±0.0a	0.0±0.0a	1.1±0.4a	0.0±0.0a	0.0±0.0a	0 (0.0%)
h^2		0.67	-0.14	0.15	---	-0.15	---

Table 4. Mean ±S.D. number of roots, root length (cm), corm damage rating, number of shoots, shoot length (cm), and number (%) of *Gladiolus* plants surviving within hardy x hardy, non-hardy x hardy, and non-hardy x non-hardy crossing groups after freezing to 0°C, -3°C, -6°C, -10°C, and -12°C and heritability (h^2) for each trait (except shoot number and plant survival) within crossing groups.

The total number of progeny with all living tissues after freezing ranged from zero (all three crossing groups at -10°C and -12°C) to 20 (26.7%; hardy x hardy at -3°C (Table 4). In general, a low range of hardy x hardy, non-hardy x hardy, and non-hardy x non-hardy progeny survived at 0°C, -3°C, and -6°C freezing temperatures (Table 4). Thus, while select progeny are hardy to -6°C, this is not within the minimum range required for herbaceous perennial survival in USDA Z4 and Z3 (-10°C and -12°C, respectively). Further breeding and testing will need to be done to determine whether or not the corms can be bred to survive lower temperatures.

4. Conclusion

A range in response was found among tested gladiolus genotypes for tissue damage after laboratory freezing (Experiment 1). 'King's Gold', for instance, was 'non-hardy' at 0°C to -10°C (dead roots/shoots). 'Great Lakes' was intermediate with living root/shoot tissues at 0, -3°C only whereas 'Lady Lucille' had living roots (0 to -6°C) and apical meristems (-3°C). LT_{50}s = -10°C for stem tissues in most genotypes; all were severely damaged or dead at -12°C. The apical meristem is more sensitive to freezing than roots, which are likewise more sensitive than stem tissue (corms) to freezing. The genetic variation (ISSRs) for the tested genotypes ranged across a wide spectrum of the gladiolus genome (Experiment 2); no correlation with ability of tissues to survive cold temperatures was found, except Sel'n. VT-03 (USDA Z3) and *G. dahlenii* (GD=0.0.86). Two principle groups separated at GD=0.53 with only one monophyletic singleton ('Great Lakes'). Sel'n. 95-49 formed a distinct group with 'Lady Lucille' and 'Gemini' (GD=0.63). No transgressive hybrid segregants for corms occurred with greater hardiness than the parents (Experiment 3). In general, crosses with ≥1 hardy parent (hardy x hardy, non-hardy x hardy) had significantly greater numbers / lengths of living roots/shoots than non-hardy x non-hardy hybrids. In all crosses at -10°C, -12°C, no living roots, root initials, apical meristems occurred. Hybrids with ≥1 hardy parent had greater numbers / lengths of living roots and shoots than the non-hardy x non-hardy group. The highest number of roots occurred in hardy x hardy crosses (at 0°C, -3°C) and non-hardy x hardy (-6°C); root lengths, in some cases (6.89 for non-hardy x hardy at -3°C), exceeded parental values. Root number is barely heritable (h^2 = 0.08) for hardy x hardy hybrids but more so (h^2=0.67) with non-hardy parents. Root length had a wider range of heritability (h^2=-0.14 to 0.37). Heritability of corm ratings is likewise low (h^2=-0.04 to 0.15).

A significantly greater number of shoots in progeny (all crossing groups) than the parents were found at the 0°C, -3°C, and -6°C temperatures, although heritability remained low (h^2=-0.43 to 0.19). While select progeny are hardy to -6°C, this is not within the minimum range required for herbaceous perennial survival in USDA Z3-4. Further breeding and selection for increased cold tolerance would be required for gladiolus to reliably survive winter conditions in northern latitudes.

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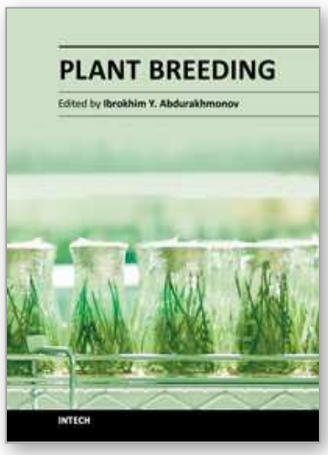

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