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Chilling relieves corm dormancy in *Calopogon tuberosus* (Orchidaceae) from geographically distant populations

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ABSTRACT

Many plant species require a chilling period to commence regrowth from overwintering structures such as buds, corms, tubers, and rhizomes. While the effects of chilling have been thoroughly studied in a horticultural context, little information exists regarding the relationship between ecotypic differentiation and chilling requirements. Effects of chilling storage organs on shoot emergence of widespread orchid species has not been examined, and ecotypic differentiation in the Orchidaceae has also received little attention. The effects of chilling on corm dormancy in *Calopogon tuberosus*, a widespread orchid of eastern North America, were studied. Seeds were collected from south Florida, north central Florida, South Carolina, and Michigan, and germinated *in vitro* to produce plants. After 20 weeks *in vitro* culture, corms were removed from seedlings and chilled for 0, 2, 4, 6, and 8 weeks. Corms were subsequently planted in a soilless potting mix and placed under *ex vitro* conditions in an environmental growth chamber. Shoot emergence was monitored bi-weekly for 16 weeks, and shoot length, leaf number, leaf width, root number, root length, and corm diameter were measured after 16 weeks. Longer chilling periods broke corm dormancy more effectively than shorter chilling treatments regardless of population. Shoots of all populations sprouted rapidly on corms after 6 and 8 weeks chilling. In addition, a higher percentage of shoots sprouted on corms after 8 weeks chilling. After 16 weeks, north central Florida and South Carolina plantlets were larger than Michigan and south Florida plantlets. Differing chilling requirements among *C. tuberosus* populations may reflect ecotypic differentiation resulting from varying environmental conditions at each site.

1. Introduction

Calopogon tuberosus var. *tuberosus* is a terrestrial orchid native to eastern North America with a large distribution from Florida to maritime Canada. Throughout its range, *C. tuberosus* occupies a variety of habitats including bogs, fens, marl prairies, and mesic roadsides. Both genetic and morphological variation have been reported recently (Goldman et al., 2004a,b; Trapnell et al., 2004), but whether ecotypes exist remains unclear. Recent studies exploring the *in vitro* ecology of seed germination and seedling development determined that photoperiod, germination media, and growing season length influenced the development of *C. tuberosus* ecotypes (Kauth et al., 2008; Kauth and Kane, 2009). Additionally, potential differences in the extent of corm dormancy among widespread *C. tuberosus* populations may be influenced by local adaptation.

Many temperate plant species form overwintering structures, such as buds, tubers, rhizomes, and corms, before unfavorable growth conditions are encountered (Garbisch et al., 1995; Rohde and Bhalerao, 2007), and remain dormant until favorable growth conditions are encountered the following growing season (Garbisch et al., 1995). In order to break dormancy, a chilling period is often required (Rohde and Bhalerao, 2007). Longer chilling periods are often required to break dormancy in tubers and corms of temperate species, but extended periods often inhibit growth and development (Clark, 1995; Yañez et al., 2005; Fukai et al., 2006). However, chilling period requirement may be different according to plant provenance in that southern species may require shorter chill periods (Perry and Wang, 1960; Garbisch et al., 1995).

Chilling requirements as a function of ecotypic differentiation have been explored in tree species (Perry and Wang, 1960; Kriebel and Wang, 1962), aquatic species (Garbisch et al., 1996), and forage grasses (Silsbury, 1961; Cooper, 1964; Eagles, 1967a,b; MacColl and Cooper, 1967). *Acer rubrum* ecotypes from Florida required no chilling to break dormancy, but longer chill periods were required to break dormancy in more northern ecotypes (Perry and Wang, 1960). *Acer saccharum* ecotypes from Georgia and Tennessee required shorter chill periods to break dormancy than ecotypes in Michigan and Ohio (Kriebel and Wang, 1962). In several forage grass species, relative growth rate of Mediterranean populations

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was higher at cooler temperatures compared to north European populations that had a higher growth rate at warmer temperatures (Cooper, 1964). Prolonged chilling decreased both survival and shoot growth of aquatic plant ecotypes from Florida (Garbisch et al., 1996).

C. tuberosus is a model orchid species to exam the relationship between chilling period and corm dormancy in widespread populations. Studying corm dormancy, chilling period necessary to break dormancy, and shoot emergence may provide insight into *C. tuberosus* ecotypic differentiation. The objectives were to: (1) assess the effect of chilling on the mechanism of corm dormancy and subsequent plantlet growth; (2) exam the response of four *C. tuberosus* populations to low temperature storage; and (3) examine ecotypic differentiation in *C. tuberosus*. Our hypotheses were: (1) all populations will require a period of chilling to break the mechanism of corm dormancy; and (2) corms from more northern populations will require a longer chilling period compared to southern populations.

2. Materials and methods

Seeds were collected from the following locations: Upper Peninsula Michigan (Menominee County, Michigan, USA), upstate South Carolina (Greenville County, South Carolina, USA), north central Florida (Levy County, Florida, USA), and south Florida (Collier County, Florida, USA). Seed capsules from all populations were collected before complete dehiscence and were stored at 23 °C over silica gel for 2 weeks. Seeds were then removed from capsules, pooled by geographic population, and stored dry in the dark at −11 °C until used. Further information about the environmental conditions for each site is supplied in supplemental Table 1.

Seeds were surface disinfected in sterile scintillation vials for 3 min in a solution of 5 mL absolute ethanol, 5 mL 6% NaOCl, and 90 mL sterile distilled, deionized water. Seeds were rinsed with sterile dd water after surface sterilization. Solutions were removed with sterile Pasteur pipettes. Seeds were transferred with a 10 μL sterile inoculating loop onto BM-1 Terrestrial Orchid Medium (*Phyto*Technology Laboratories, Shawnee Mission, KS) contained in 100 mm × 15 mm Petri plates. The medium was supplemented with 1% activated charcoal. Medium pH was adjusted to 5.7 with 0.1 N KOH prior to autoclaving for 40 min at 117.7 kPa and 121 °C. Ten replicate Petri plates with 30 mL medium each were used for each seed source with approximately 100 seeds per plate. Cultures were placed in an environmental growth incubator (#I-35LL; Percival Scientific, Perry, IA, USA) under cool-white fluorescent lights in a 12 h photoperiod at 24.2 ± 0.2 °C and 40 μmol m^{-2} s^{-1}.

After 8 weeks culture, seedlings were transferred to larger culture vessels for further growth and development. Nine seedlings were transferred to individual *Phyto*Tech Culture Boxes (*Phyto*Technology Laboratories, Shawnee Mission, KS) containing 100 mL of BM-1 Terrestrial Orchid Medium. Five replicate vessels were prepared for each treatment and seed source combination for a total of 45 seedlings per treatment. A total of 25 vessels with a total of 225 seedlings were prepared for each seed source. A total of 900 seedlings were transferred. Seedlings grew *in vitro* for another 12 weeks, for a total of 20 weeks culture. Environmental conditions were the same as described previously.

After the 20 weeks, shoots and roots on seedlings were removed so that only corms remained. The nine corms in each *Phyto*Tech box were transferred to Sigma Phytatrays I (#P1552, Sigma–Aldrich, St. Louis, MO) containing 100 mL of moist, sterilized vermiculite. Five Phytatrays I (114 mm × 86 mm × 63.5 mm) were prepared for each treatment. Cultures containing the corms were subsequently stored at 10 ± 0.3 °C for 2, 4, 6, and 8 weeks in complete darkness; a control (no cold storage) was also used. Five culture vessels per seed source were allocated to each chilling period.

The 20 culture boxes (five replications per each of the four populations) allocated to the five chilling treatments were subsequently removed after the chilling period. Corms were subsequently planted in a 9-cell pack containing Fafard 2 (Conrad Fafard, Inc., Agawam, MA, USA). Corms were planted in a randomized complete block design with block designated as the chill treatment so that block 1 was the control, etc. Each seed source was allocated to each block, and blocks were replicated five times. Corms were buried approximately 1 cm below the soil line. Trays were placed in a walk-in growth chamber under a 16/8 h L/D photoperiod at 27 ± 2.2 °C and an average relative humidity of 85%. Four 400-W metal halide bulbs (Sylvania, Danvers, MA, USA) provided a light level of 167 μmol m^{-2} s^{-1}. Corms were watered as needed and as frequently as daily.

Shoot emergence date was recorded by the presence of the new shoot emerging from the soil. Every 2 weeks, starting upon emergence and continuing until week 16, shoot length was measured from the soil surface to the shoot apex. At the final data collection, leaf number, leaf width, shoot height, root number, root length, corm diameter, and axillary shoot formation were recorded. Axillary shoots (Fig. 1) grow from storage organs to form new storage organs (Dixon and Pate, 1978; Hollick et al., 2001). Percent shoot emergence and percent survival, noted by the presence of a corm beneath the soil surface, were recorded. Logistic regression was used to assess the affect of chilling treatment and population on percent shoot emergence, percent survival, and percent axillary shoot formation using the generalized linear mixed model procedure (proc glimmix macro) in SAS v9.1. Least-square means (lsmeans) were used to assess mean separation. Endpoint measurement data were analyzed using the general linear procedure (proc glm), ANOVA (see supplemental Table 2), and least-square means in SAS v9.1.

3. Results

3.1. Effects of chilling on shoot emergence

The main effects (population and chilling period), and their interaction all significantly influenced the number of days to shoot emergence. The average number of days to shoot emergence post-chilling was less under the longer chill periods of 6 and 8 weeks, regardless of population (Fig. 2A). Corms subjected to the control or the 2-week chilling period exhibited the slowest shoot emergence. South Carolina and south Florida corms chilled longer than 6 weeks exhibited the quickest shoot emergence. Michigan corms required 4 weeks or longer for quickest shoot emergence, while shoots emerged faster when north central Florida corms were chilled for 8 weeks (Fig. 2A).

Percent shoot emergence was highly influenced by the main effect of chilling period, but the main effect of population and the interaction between chilling treatment and population were not significant. Lower percent shoot emergence was observed when corms were chilled for shorter periods (Fig. 2B). Less than 20% shoot emergence was observed in unchilled corms and following the 2-week chilling period among all populations. In fact, only one shoot from South Carolina emerged in the control and only one shoot from north central Florida emerged in the 2-week chilling treatment. Chilling periods longer than 6 weeks provided the highest percent shoot emergence in Michigan corms (Fig. 2B), while 8 weeks chilling provided the highest shoot emergence for all other populations. Approximately 90% shoot emergence occurred on South Carolina, north central Florida, and south Florida corms, compared to 78% for Michigan corms (Fig. 2B).