Chilling Requirements to Break Dormancy of *Veratrum californicum*

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Abstract. *Veratrum californicum*, a native of the western United States, has long been used in herbal medicine and now also has potential pharmaceutical uses. As a result of a projected increasing demand for *V. californicum* biomass for pharmaceutical purposes, the development of a chilling protocol for enhanced cultivation efficiency is needed. To study the effects of chilling on the growth of *V. californicum*, field-collected rhizomes with attached bulbs and roots were potted, stored at 10 °C for 2 weeks, and subsequently chilled at 5 °C for 30 to 180 days before transfer to a greenhouse or growth room. Twenty plants were transferred to the greenhouse every 30 days to observe growth. Ten plants were harvested at shoot emergence and the remaining 10 when leaves were fully expanded. In addition, 10 plants were transferred from 5 °C to a growth room every 30 days where net photosynthetic rates were measured. Longer chilling duration correlated with a reduction in days to shoot emergence and leaf expansion. The net photosynthetic rates of *V. californicum* plants chilled for 120, 150, or 180 days were higher than those of plants chilled for only 30, 60, or 90 days. Plants exposed to longer chilling durations were taller and had larger, more numerous leaves. Interestingly, *V. californicum* shoot emergence was also observed in the dark at 5 °C after the bulbs had been stored for 210 days. Growth of the root systems of plants was also observed during chilling. In conclusion, chilling was necessary at 5 °C for a minimum of 120 days to force early emergence and vigorous growth of *V. californicum*.

Veratrum californicum Durand (corn lily) is an herbaceous perennial monocot native to wet meadows across much of western North America [USDA NRCS (Natural Resources Conservation Service), 2011]. *Veratrum californicum* has long been used in herbal medicine and now also has potential pharmaceutical applications. In recent years, the *V. californicum*-derived phytochemical cyclopamine and its derivatives have been explored as promising therapeutic agents for the treatment of tumors arising from activation of the Hedgehog signaling pathway (Berman et al., 2002; Chen et al., 2002; James et al., 2004; Taipale and Beachy, 2001; Tremblay et al., 2009). To meet the projected pharmaceutical demand for this alkaloid, a dependable cultivation system will be required. To that end, we are developing cultivation protocols for the greenhouse production of *V. californicum*.

Extensive field surveys were conducted to select planting stock with high concentrations of cyclopamine and related alkaloids. An ecotype of primary interest was identified in numerous high-elevation bogs and meadows in Utah and Idaho where plants have had the highest yields of alkaloids over several successive years of observation (unpublished data). In these higher (above 2400 m) regions with native *V. californicum* populations, bulbs undergo prolonged (seven- to eight-month) periods of winter dormancy under a deep snow pack.

Average daily soil-temperature data collected between 2003 and 2010 at three depths (5, 20, and 51 cm) from several independent USDA weather station locations in Utah proximal to natural *V. californicum* populations of interest show that soil temperatures typically drop below 10 °C by 1 Oct. and do not return above 10 °C until after 1 May. Field observations report a rapid burst of vegetative growth after the recession of the snow line. Taylor (1956) reported that a period of exposure to cold temperatures is necessary to permit successful vegetative growth of *V. californicum* and that this prolonged exposure to cold is essential for successful cultivation. Dormancy is defined as a lack of growth because specific conditions have not

been met (e.g., a period of low temperature). We anticipate that a successful production protocol for *V. californicum* collected from natural populations in temperate regions, sites above 2400 m in elevation, will include defined chilling treatments.

In this study we report the influence of varied periods of chilling on shoot emergence and growth using mature, field-collected *V. californicum* plants. Our goal was to force *V. californicum* to emerge before a hypothetical natural period of snow cover as a means of shortening the production cycle. Thus, we determined the minimum length of cold treatment required to break dormancy and whether shortened dormancy affected shoot emergence and vigor. Growth of the shoot systems was also observed in both greenhouse and artificially illuminated growth rooms.

Materials and Methods

Chilling. A few hundred plants of *Veratrum californicum*, rhizomes with attached bulbs and roots, were selected for uniformly large size from a wild population of thousands dug in a meadow ≈ 1000 m² (mechanically harvested for the purpose of pharmacological research) in Boulger Canyon, UT (lat. 39°36' N, long. 111°13' W, elevation 2671 m). This site was chosen because the planting stock from which the plants were collected had high concentrations of cyclopamine and related alkaloids (unpublished data). The next day, 14 Sept. 2010, plants were shipped overnight to Clemson, SC, and on arrival, the rhizomes, each with one bulb, were sorted into two size groups based on bulb circumference [12.2 ± 1.0 cm (mean $\pm$ SD) and 10.2 ± 1.4 cm for large and small bulb sizes, respectively] and potted into 7.6-L (large) or 3.8-L (small) plastic containers filled with Fafard 3B mix [45% Canadian sphagnum peatmoss, 25% processed pine bark, 15% perlite, 15% vermiculite, starter nutrients (40 to 230 mg·L⁻¹ nitrogen; 5 to 30 mg·L⁻¹ phosphorus; 40 to 200 mg·L⁻¹ potassium, calcium, and sulfur; 25 to 80 mg·L⁻¹ magnesium), wetting agent, dolomitic limestone; Conrad Fafard, Inc., Anderson, SC]. Each plant was drenched with 330 ppm Subdue® [25.1% Metalaxyl: *N*-(2, 6-dimethylphenyl)-*N*-(methoxyacetyl) alanine methyl ester, 74.9% inert ingredients; Syngenta Crop Protection, Inc., Greensboro, NC] to prevent root rot.

Veratrum californicum plants were first stored in the dark at 10 °C and 70% relative humidity for 2 weeks (pretreatment) and then chilling treatments were initiated at 5 °C and 65% relative humidity for 30, 60, 90, 120, 150, and 180 d in a controlled environment room (Model# 120-208; Climate Technologies, Inc., Laytonville, MD) in the Clemson University Biosystems Research Complex. Bulbs and rhizomes not included in these experiments were handled in the same manner as experimental units and were retained under experimental conditions for 220 d. All bulbs and rhizomes were watered at 2-week intervals and substrate volumetric water content was maintained above $44.2\% \pm 5.8\%$ as measured

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using Decagon 10-HS soil moisture sensors (Decagon Devices, Inc., Pullman, WA).

Greenhouse and growth room forcing. Nineteen plants from each bulb size treatment were removed from the cold environment room on a monthly basis. Four plants were destructively harvested for analysis (harvest described subsequently), 10 plants transferred into a greenhouse, and five plants transferred into a growth room. Both the greenhouse and growth room are located in the Clemson University Biosystems Research Complex.

Plants transferred to the greenhouse environment were fertigated with 100 mg·L⁻¹ nitrogen CalMag special fertilizer (15N–2.2P–12.5K; Scotts Peters Excel, Marysville, OH). Substrate volumetric water content was measured by using Decagon 10-HS soil moisture sensors and maintained at or above 48.5% ± 4.9%. An ECD DataWorker (ECD, Inc., Milwaukie, OR) recorded canopy air temperature on an hourly basis. Ambient air temperatures in the greenhouse were 20.2/17.0 ± 6.5/5.2 °C day/night during the experimental period. Light intensity at the canopy level was monitored hourly with LI-190 Quantum sensors (LI-COR® Biosciences, Lincoln, NE) and a LI-1400 data logger (LI-COR® Biosciences). The average daily light integral over the experiment period was ≈23.3 mol·m⁻²·d⁻¹.

Plants in the growth room (3 m × 3 m) were fertigated with 100 mg·L⁻¹ nitrogen CalMag special fertilizer every other day, and substrate volumetric water content was maintained above 41.2% ± 5.3%. Ambient air temperature in the growth room was 23.1 ± 0.7 °C during the light and dark period. Light, during a 12-h photoperiod, was provided with 1000-W metal halide lamps (Agrosun Gold; Hydrofarm, Inc., Medley, FL), which were placed 1.5 m above the bench. Light intensity at the plant canopy surface was 16.0 ± 0.6 mol·m⁻²·d⁻¹. Relative humidity in the growth room was maintained at 43.1% ± 8.2% by using an Argus humidity controller (Argus Control Systems Ltd., British Columbia, Canada).

Data collection. Times to shoot emergence (≈2 to 5 cm long bud emergence from bulb) and leaf expansion (a minimum of three fully expanded leaves) were recorded for plants in both bulb size treatments in the growth room (shoot emergence n = 5; leaf expansion n = 5) and greenhouse (shoot emergence n = 10; leaf expansion n = 5). The time to shoot emergence and leaf expansion began (Day 0) when plants were moved from the chiller (i.e., the end of treatment) into their growth environment. In the greenhouse, five *V. californicum* plants from each bulb size treatment were harvested at shoot emergence and at leaf expansion. At harvest, the number of newly developed roots was counted. Plant parameters measured once plants reached maturity, defined as the end of leaf expansion, included plant height (cm) from the bulb basal plate to the top of the stalk, the number of leaves per plant, and length and width (cm) of five leaves counted

upward from the second most mature leaf at the base of the stalk. At the end of the experiment (24 May), dead plants were counted.

Photosynthetic light response curves were recorded for all plants surviving after 40 d in the growth room by using a CIRAS-2 portable photosynthesis system with an integrated Chlorophyll Fluorescence Module (CFM) unit (PP Systems International, Inc., Amesbury, MA) mounted with an automatic universal PLC6 broad leaf cuvette. All plants were well watered before measurements. The third, fourth, or fifth fully expanded leaf, counting from the top of the plant downward, was chosen for the measurements. Two of the three leaves per plant were recorded. The number of plants measured per chilling period for which photosynthetic light response curves were recorded include one, five, nine, 10, nine, and eight from 30, 60, 90, 120, 150, and 180 d of chilling, respectively. The CO₂ concentration within the leaf chamber was maintained at 375 μmol·mol⁻¹, whereas the photosynthetic light response curve was measured. Constant temperature (25 °C) and relative humidity (75%) were maintained within the leaf cuvette during each measurement. Before each response curve was measured, the leaf clamped inside the leaf cuvette was exposed to a dark adaptation period of 30 min. Light intensities were gradually increased from 0 to 2000 μmol·m⁻²·s⁻¹ at 200 μmol·m⁻²·s⁻¹ light intervals. The minimum holding time between each step was 45 s.

Statistical analysis. Plants were assigned to treatments in a completely randomized design. Data were analyzed with JMP Version 9.0 (Statistical Analysis System, Cary, NC). Main effects and interactions among chilling duration, bulb size, and/or location/stage were analyzed using a three-way analysis of variance. Each plant was considered as an experimental unit. Linear and quadratic trend analyses were also conducted.

Results and Discussion

Shoot emergence, leaf expansion, and root regeneration. Chilling duration significantly affected shoot emergence and leaf expansion ($P < 0.0001$) irrespective of bulb size or growing condition (growth room or greenhouse). As the duration of chilling increased, the time required for shoot emergence (Fig. 1A) and leaf expansion (Fig. 1B) decreased. Langens-Gerrits et al. (2003) also observed that shoot emergence from dormant bulbets of *Lilium speciosum* 'Rubrum' No. 10¹ occurred more quickly with greater uniformity after a longer chilling (6 weeks vs. 4 weeks) duration at 5 °C. Before 120 d of chilling, it took shoots and leaves a prolonged time to emerge and expand (Figs. 1A–B). These data suggest that longer (greater than 120 d) chilling periods may not significantly affect days to shoot emergence and leaf expansion. However, the quality of plant growth (visual observations) continued to improve with longer chilling periods. None of the plants in our study flowered. Flowering in *V. californicum*

Fig. 1. Effect of chilling duration at 5 °C on *Veratrum californicum* shoot emergence (A) and leaf expansion (B). Vertical bars represent the SEM observed for shoot emergence (n = 30) and leaf expansion (n = 20). Data were pooled by bulb size and growing condition because no significant differences were noted.

Fig. 2. The effect of chilling duration at 5 °C on percent survival of *Veratrum californicum* plants grown in a temperature-controlled growth room (n = 10, per chilling treatment duration).

is sporadic (Taylor, 1956), and the potential to flower may be predetermined during the preceding season of growth.

New roots began to form during the first 30-d chilling treatment, and root formation continued over the entire range (30 to 180 d) of chilling treatments. New primary roots formed on 10.3% (19 of 184) of bulb plates; there was an average of four roots per bulb, which averaged 5 cm in length. New secondary roots also emerged from the old roots on 88.6% of bulbs (163 of 184). Chilling duration significantly affected the number of new secondary roots per bulb ($P = 0.002$) with more secondary roots forming on large-sized bulbs ($P = 0.0002$). Additional roots formed after the chilled bulbs were moved into the greenhouse environment ($P < 0.0002$).

Pak et al. (1995) previously reported that water uptake was facilitated by root growth during chilling and that this was necessary for shoot emergence in onions.

Plant performance in the growth room. Fifty percent of the *V. californicum* plants transferred into the growth room died before the conclusion of the experiment. Among the dead plants, only three plants had a few new secondary roots when examined (data not shown); thus, the lack of root development or loss or new roots may have contributed to the observed high mortality rate. Longer durations

of chilling correlated positively with increased plant survival ($P = 0.04$; Fig. 2). Constant day and night temperatures in the growth room may have also reduced plant vigor, because the *V. californicum* ecotype studied is adapted to a region with considerably lower night temperatures during the growing season, as observed at proximal field weather stations.

Shoots of *V. californicum* were larger after longer durations of chilling. Plant height, the number of leaves, and leaf length and width increased with longer chilling duration

($P < 0.0001$; Fig. 3). Although leaf size (length and width) increased with chilling durations of greater than 120 d, the number of leaves did not increase significantly. The maximum effect of chilling on plant height was observed in durations of greater than 150 d. These results indicate that shoot buds are prepared not only for emergence, but also for subsequent plant growth (leaf expansion) during the chilling period. Langens-Gerrits et al. (2003) reported that 5 °C cold treatment enhanced size and mass of non-dormant *Lilium speciosum* Thunberg. 'Rubrum No. 10' bulblets compared with non-treated plants. Shin et al. (2002) reported that the physiological dormancy of *Lilium* 'Casablanca' and *Lilium* 'Mona' bulblets was released more rapidly with greater shoot production after a 9-week cold treatment at 4 °C as compared with 9 weeks at either 10 or 25 °C. Although our results indicate that the chilling period influenced the number of leaves, it is possible that the inadequate root mass of plants in chilling treatments of less than 120 d also may have decreased the potential number of leaves ultimately expanded from the bulb. *Veratrum californicum* is a facultative wetland plant species and the root system was impacted when lifting plants from the field for our study. Thus, it is possible that inadequate root formation limited water uptake and bulb growth during shorter chilling durations.

The potential net photosynthetic rates of *V. californicum* plants increased during the first 120 d of chilling. The net photosynthetic rates of leaves emerging from *V. californicum* plants chilled for 30, 60, and 90 d ($P = 0.2$) did not differ significantly from each other. Nor were there differences in the net photosynthetic rates of plants chilled for 120, 150, and 180 d ($P = 0.99$). Thus, the data were pooled into two groups. One group (inadequate chilling) included the net photosynthetic rates of plants chilled for 30 to 90 d. The other group (adequate chilling) included plants grown from bulbs chilled for 120 to 180 d. Plants chilled adequately had significantly higher net photosynthetic rates than did those with inadequate chilling ($P < 0.0001$). The adequately chilled plants had greater root mass and higher net photosynthetic rates; these attributes may be the result of more massive sink. Sink strength is considered a product of sink size and sink activity (Ho, 1988).

The maximum net photosynthetic rate of plants with adequate chilling was twice that of plants with inadequate chilling (Fig. 4). Plants chilled for more than 210 d emerged in the cold storage room in the dark (non-forced growth) and had similar photosynthetic rates to plants with adequate chilling periods once shoots were established. These data are consistent with field observations of the Boulger Canyon *V. californicum* ecotype, which can sprout beneath the snow pack under similar environmental conditions (0 to 5 °C) as those maintained in the growth chamber (observations in Mammoth-Cottonwood, UT, near Boulger Canyon).

Fig. 3. Chilling duration at 5 °C influenced *Veratrum californicum* height (A), number of leaves (B), length (C), and width (D) of leaves after 40 d of subsequent growth in a temperature-controlled growth room at 23 °C. Vertical bars represent the SEM response of $n = 8$ plants for the 30 and 60 d chilling treatments and $n = 10$ plants for the 90, 120, 150, and 180 d chilling treatments.

Fig. 4. The influence of chilling duration at 5 °C on the net photosynthetic rate of *Veratrum californicum* plants after 40 d of subsequent growth in a temperature-controlled growth room at 23 °C. Data for plants chilled for 30, 60, and 90 d were pooled (inadequate), and data for plants chilled for 120, 150, and 180 d were also pooled (adequate). Non-forced plants emerged in the dark after greater than seven months of chilling. Vertical bars represent the SE of the average net photosynthetic rate of inadequate ($n = 15$), adequate ($n = 27$), and non-forced ($n = 4$) plants.

In conclusion, adequate chilling plays a critical role in *V. californicum* growth. A greenhouse production protocol for *V. californicum* will likely need to incorporate a minimum of 120 d of chilling at 5 °C as part of the growth cycle. Roots grow during chilling and greater root mass influences subsequent shoot performance. Although our data indicate that maximal photosynthetic rates of plants grown from bulbs chilled for 120, 150, or 180 d were similar, plant quality parameters differed, and plants with longer chilling durations appeared to be larger and of higher quality as observed by overall appearance. The influence of chilling duration on plant mass and alkaloid production should be examined, because the ultimate purpose of developing a production protocol for *V. californicum* is to develop an alkaloid production system to support pharmaceutical use.

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