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Gender-specific seasonal pattern and altitudinal variation in freeze tolerance responses of Seabuckthorn (*Hippophae rhamnoides* L.)

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Abstract.
BACKGROUND: In dioecious species the morphological and physiological adjustment to cold and freezing conditions may differ significantly between male and female individuals due to greater reproductive effort by females.
OBJECTIVES: To assess the importance of gender-specific responses of *H. rhamnoides* of cold acclimation and freeze tolerance in trans-Himalayan environments.
METHOD: We measured the proline contents in leaves and shoots in male and female *H. rhamnoides* from mid August to mid December in standing crop.
RESULTS: Proline content in leaves showed a significant increasing trend from August to October followed by a steady decline from November onwards in both the genders. Progression in season from August to December is related linearly to the increase in proline contents in both male ($R^2 = 0.967$) and female ($R^2 = 0.926$) shoots. Increase in altitude (3202–3812 m amsl) of plant origin is related linearly to increase in proline contents in both male ($R^2 = 0.676$) and female ($R^2 = 0.858$) shoots. The overall proline contents in both leaves and shoots were significantly higher in male (112 ± 77, 143 ± 66 μM g^{-1}, respectively) than in female (87 ± 46, 119 ± 82 μM g^{-1}, respectively).
CONCLUSION: The study suggested sexually dissimilar responses to cold and freezing conditions in *H. rhamnoides* and that male possess a better self protection mechanism than female. Leaves developed tolerance against cold stress more quickly than shoots.

Keywords: Abiotic stress, acclimation, chilling, climate change, himalaya, proline

1. Introduction

Woody plants in temperate regions are subjected to large seasonal variations of temperature. Plants adapt to such conditions by evolving annual growth cycle that alternates between active shoot growth and vegetative dormancy closely synchronized with the seasonal changes [1]. Most plant species have evolved a degree of cold tolerance, the extent of which is typically dependent on combination of the minimum temperature experienced and the length of exposure to cold stress [2]. Overwintering temperate plant species such as Seabuckthorn (*Hippophae rhamnoides* L.) are able to resist low temperature and freezing. Generally, in these species the level

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of freezing tolerance is season-dependent and can be modulated by a prior period of acclimation (pre-hardening) at low non-freezing temperatures, during which time a number of morphological, physiological, and molecular changes occur [3]. The full degree of tolerance (hardening) is achieved thereafter when plants are exposed to a period of sub-zero temperature. It is well documented that accumulation of low-molecular weight compounds is observed during exposure to low temperature. Changes in water-soluble carbohydrate [4] or in free amino acids, especially proline [5, 6], are associated with cold acclimation and acquisition of frost tolerance. It is suggested that proline might be involved in osmoregulation and in the protection of proteins against dehydration [7], membrane stabilization [8] or regulation of certain enzymatic systems [9] during low temperature stress.

The ability of plants to adapt to and survive freezing temperatures has many facets, which are often species specific, and is the result of the response to many environmental cues, rather than just low temperature [10]. However, most of the freeze tolerance responses in plants are studied in controlled conditions in growth chambers. There are significant differences between natural and artificial cold acclimations. Plants which have been cold acclimated in growth chambers may respond differently than those acclimated naturally [11, 12]. Varying diurnal temperatures that produce mixed signals in the field are in contrast to constant temperatures in a growth chamber. Any research designed to explore and understand cold hardiness should be verified in the context of the physiology, growth habit and life cycle of the plant grown under natural conditions [10].

In dioecious species the morphological and physiological adjustment to cold and freezing conditions may differ significantly between male and female individuals due to greater reproductive effort by females. The cost of reproduction involves prioritization of resources in fruit development rather than in vegetative growth or protection in females. A major investment in reproduction is generally associated with the disadvantage in terms of oxidative stress and cellular injuries, particularly under adverse conditions [13]. Not accounting for sexual variation could lead to incorrect assessment of a species response to frost. However, to the best of our knowledge no studies have been conducted that have addressed the relative importance of the gender of the plant for freeze tolerance in natural conditions particularly in the trans-Himalaya.

Hippophae rhamnoides L. is an ecologically and economically important dioecious plant. It is found in a large altitudinal range, from the sea shores in Europe to over 4694 m in trans-Himalayan Ladakh. Development of freeze tolerance is strongly influenced by gender and ecotype in this species [14]. Studies conducted in controlled conditions where plants were exposed to cold stress conditions (4°C for 0–24 h) suggested that males are more responsive to exposure to low temperature, and resulted in earlier cold acclimation and higher freeze tolerance than females [14, 15]. However, freeze tolerance of *H. rhamnoides* in natural conditions has not been investigated in concert with measurements of their progeny in common garden experiments. *H. rhamnoides* are easy to propagate by stem cuttings, and the availability of clonal material facilitates the testing of identical genotypes under different conditions. *H. rhamnoides*, therefore, presents an excellent opportunity to investigate the relative contributions of environmental and gender factors to the relationship between freeze tolerance and altitude. Therefore, the aim of this study was to assess the importance of gender-specific responses of *H. rhamnoides* on cold acclimation and freeze tolerance in trans-Himalayan environments. Proline contents were analyzed to use as a marker for freeze tolerance in standing crop in natural conditions. The research included two components: (a) field studies along an altitudinal gradient, and (b) common-garden approach, in which a large number of cuttings from several male and female shrubs collected along an altitudinal gradient were planted in an experimental plot. We expected to find gender differences and strong altitudinal variation for freeze tolerance.

2. Materials and Methods

2.1. Study site

We collected *H. rhamnoides* subsp. *turkestanica* from seven natural sites along an altitudinal gradient from 3202 to 3812 m amsl in trans-Himalayan Ladakh region. Common-garden experiment was carried out at an experimental farm (34°08.2'N; 77°34.3'E, 3350 m amsl) on a horizontal site with direct sunshine at Defence Institute of High Altitude Research (DIHAR) in trans-Himalayan Ladakh, India. The mean maximum and minimum temperature during 2014 and 2015 recorded at DIHAR was 12.9 ± 8.8°C and -0.2 ± 9.0°C, respectively. The mean monthly temperature was highest in July (25.6°C), and lowest in January (–13.2°C). The mean maximum and minimum relative humidity was $31.0 \pm 4.3\%$ and $24.7 \pm 3.7\%$, respectively. The average annual precipitation was 163 mm.

2.2. Leaf and shoot materials

In December 2014, we collected a single branch from 10 male and 10 female adult *H. rhamnoides* at each site in natural field condition. All branches were collected on the sunny side of the shrub. Fully grown leaf and shoot samples were collected from each branch for measurement of proline contents. Between 04 and 14 April 2015, dormant cuttings of pencil thickness were taken from each shrub and planted at an experimental farm at DIHAR. Fully expanded leaf and shoot samples were collected on 15th of every month from August to December 2015 from each of the rooted plants for measurement of proline contents in garden-grown plants.

2.3. Proline contents

The proline contents was determined using the method described earlier [16]. Proline was extracted from 0.5 g fresh weight plant material homogenized in 10 ml of 3% aqueous sulfosalicylic acid and the homogenate was filtered through Whatman # 2 filter paper. 2 ml of filtrate was mixed with 2 ml acid-ninhdrin and 2 ml of glacial acetic acid in a test tube for 1 h at 100°C, and the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene, mixed vigorously for 15–20 sec. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and absorbance at 520 nm was recorded in a micro-plate reader (SpectroMax M2 e, Molecular Devices, Sunnyvale, CA, United States) using toluene for the blank. The proline concentration was determined from a standard curve and calculated on fresh weight basis as follows:

[(μg proline/ml × ml toluene)/115.5 μg/μmole]/[(g sample)/5]=μmoles proline/g fresh weight material.

2.4. Statistical analysis

In all the experiments each data point was the mean of three replicates and comparisons with P-values ≤ 0.05 were considered significantly different. All statistical analysis were performed using SPSS (Statistical Program for Social Sciences, SPSS Corporation, Chicago, Illinois, USA) The values for the parameters were subjected to one-way analysis of variance and the mean differences were compared by *post hoc* analysis with 2-sided Tukey's HSD to check significant differences between different months. Student's t test was used to compare significant difference between male and female in each month.

3. Results

3.1. Gender-specific seasonal pattern in freeze tolerance

Seasonal pattern in acclimation and freeze tolerance is presented in Table 1. Proline contents in leaves and shoots varied significantly during the sampling period. The proline contents in leaves showed a significant increasing trend from August to October followed by a steady decline from November onwards (Table 1) in both gender. However, the shoot proline contents showed a steady increasing trend from August to December.

Table 1
Seasonal pattern in proline contents as a measure of cold hardiness in *Hippophae rhamnoides* originated from different altitude in year 2015

Tissue	Date	Proline Contents (μM g^{-1}FW)	
		Male	Female
Leaves	15 Aug	0.3 ± 0.2^{a}	0.3 ± 0.2^{a}
	15 Sept	26.8 ± 17.1^{b}	32.7 ± 23.5^{ab}
	15 Oct	$234.3 \pm 76.0^{d***}$	130.7 ± 38.9^{cd}
	15 Nov	71.3 ± 23.5^{c}	69.1 ± 20.9^{b}
	15 Dec	46.9 ± 13.7^{bc}	31.8 ± 7.1^{ab}
Shoot	15 Aug	0.6 ± 0.4^{a}	0.5 ± 0.4^{a}
	15 Sept	88.1 ± 72.0^{c}	91.0 ± 62.7^{bc}
	15 Oct	$219.2 \pm 135.9^{d***}$	108.5 ± 77.7^{bc}
	15 Nov	$237.5 \pm 113.1^{d*}$	164.0 ± 120.4^{c}
	15 Dec	$361.7 \pm 158.6^{d*}$	298.2 ± 132.5^{d}

Values represented as mean ± SD. For each column, different lowercase letters indicate significantly different at $p \leq 0.05$, as measured by 2-sided Tukey's HSD. *value significantly higher than that of opposite sex at $p \leq 0.01$, ***value significantly higher than that of opposite sex at $p \leq 0.001$.

Table 2
Minimum temperature recorded at night at the experimental site in trans-Himalayan region (3350 m amsl) in year 2015

Period	Mean temperature (°C)		Number of days minimum temperature recorded at			
	Min	Max	Above 16°C	15 to 6°C	5 to 0°C	Below –1°C
16 Jul-15 Aug	12.5 ± 1.9	26.0 ± 2.2	2	29	0	0
16 Aug-15 Sept	9.1 ± 3.1	22.1 ± 2.5	0	27	4	0
16 Sept-15 Oct	3.0 ± 2.7	16.6 ± 2.3	0	5	25	0
16 Oct-15 Nov	–2.6 ± 3.4	10.5 ± 2.8	0	0	8	23
16 Nov-15 Dec	–8.3 ± 3.7	6.1 ± 3.7	0	0	0	30

Progression in season from August to December is related linearly to the increase in proline contents in both male ($R^2 = 0.967$) and female ($R^2 = 0.926$) shoot. Leaves of male plants had significantly higher proline contents (234 ± 76 μM g^{-1}) than female (131 ± 39 μM g^{-1}) in October ($P \leq 0.001$, Student's *t*-test). However, in shoot the proline contents remained significantly higher in male than female from October to December (Table 1). Throughout the sampling period the proline content in shoots remained significantly higher than that in leaves except in October. Acclimation began in September with significant increased in proline contents in both leaves and shoots. Four incidents of temperatures between 0°C to 5°C occurred during the period (Table 2).

3.2. Altitudinal variation in freeze tolerance

Altitude of plant origin did not have a significant impact on leaves proline contents in both male and female *H. rhamnoides* (Table 3). No increasing or decreasing trend was observed in leaves proline contents with increasing altitude (Table 3). However, increasing altitude is related linearly to increase in proline contents in both male ($R^2 = 0.676$) and female ($R^2 = 0.858$) shoot. Males collected from 3340 m altitude contained significantly higher proline in both leaves and shoot than those of females ($P \leq 0.01$, Student's *t*-test). The overall proline contents in both leaves and shoot was significantly higher in male (112 ± 77, 143 ± 66 μM g^{-1}, respectively) than female

Table 3

Altitudinal variation in proline contents in leaves and shoots as a measure of cold hardiness in *Hippophae rhamnoides* in December 2014 under natural field condition

Altitude (m asl) of origin	Proline Contents ($\mu M\ g^{-1}$ FW)			
	Leaf		Shoot	
	Male	Female	Male	Female
3203 ± 5.6	67.4 ± 23.6^{a}	64.9 ± 23.6^{a}	84.2 ± 43.4^{a}	59.6 ± 56.6^{a}
3239 ± 5.0	145.8 ± 103.7^{ab}	133.4 ± 61.4^{b}	113.6 ± 48.2^{ab}	73.0 ± 43.6^{a}
3260 ± 4.6	67.6 ± 34.5^{a}	64.1 ± 23.8^{a}	121.3 ± 47.4^{ab}	96.6 ± 66.1^{abc}
3340 ± 8.7	$177.5 \pm 108.0^{b**}$	65.7 ± 30.3^{a}	$175.1 \pm 75.8^{b***}$	75.0 ± 19.3^{ab}
3464 ± 23.9	119.3 ± 23.7^{ab}	101.4 ± 34.0^{ab}	180.5 ± 65.9^{b}	163.8 ± 53.8^{bcd}
3636 ± 49.6	69.2 ± 25.4^{a}	66.0 ± 24.8^{a}	165.0 ± 64.4^{b}	166.3 ± 98.8^{cd}
3812 ± 24.8	134.6 ± 86.1^{ab}	113.1 ± 55.6^{ab}	159.6 ± 59.0^{ab}	195.7 ± 90.8^{d}
Mean	$111.6 \pm 77.4^{**}$	86.9 ± 46.0	$142.9 \pm 65.7^{***}$	118.6 ± 81.5

Values represented as mean ± SD. For each column, different lowercase letters indicate significantly different at $p \leq 0.05$, as measured by 2-sided Tukey's HSD. **Value significantly higher than that of opposite sex at $p \leq 0.01$.

(87 ± 46, $119 \pm 82\ \mu M\ g^{-1}$, respectively). Within each sampling site, proline content was significantly lower in leaves than shoot irrespective of the gender. However, the opposite was observed in samples collected from 3239 ± 5 m amsl.

4. Discussion

H. rhamnoides has a great ability to withstand different environmental stresses. In this study, male and female *H. rhamnoides* were examined for proline accumulation during the process of cold acclimation and developing freeze tolerance. The study was conducted during their transition from active growth to dormant stage in field experiment running from August to December in natural conditions. Leaves and shoots were monitored in an attempt to characterize proline contents during the cold acclimation and frost tolerance process. The plants were able to acclimate to cold, as shown by increased proline contents, when exposed to cold stress. Acclimation began in September with significant increased in proline contents in both leaves and shoots. Four incidents of temperatures between 0°C to 5°C occurred during the period (Table 2). Different woody perennials acclimate differentially within a given temperature range [17]. In *Weigela* a range of cultivars acclimate late, with substantial hardening taking place concurrent with the minimum air temperature dropping below 5°C on several occasions [18]. Likewise, in two populations of *Leptospermum scoparium* the apparent threshold temperature for the onset of frost hardening was about 6°C [19]. Exposure of *Rhododendron* to 5°C in controlled condition is reported to confer cold tolerance [20]. In this study cold hardening occurred during mid October when temperatures below 5°C were observed on 25 days between mid September and mid October as marked by significant increase in proline contents in both leaves and shoots. Sub-zero temperature does not seem to be a prerequisite for hardening in *H. rhamnoides*. From mid October onwards sub-zero temperature was a common phenomenon and proline contents in shoot remained significantly high.

In this study, it was observed that plants exposed to temperature 0 to 5°C during mid September to mid October had significantly higher proline contents in both the sexes; however, males had much more proline contents than females. Increased proline contents in cold-stressed male suggest that males have a better osmoregulation mechanism than females, because proline are the major osmoregulation substances in the expanded leaves of many plants [21]. Proline is vital in preventing protein denaturation, a source for carbon and nitrogen, and for acting as a hydroxyl radical scavenger [22, 23]. The increased proline found in male is a sign of better protection against environmental stress in male cells compared to female cells. These results suggest that response to cold and freezing by gender is significant and that males possess a better self protection mechanism than females in *H. rhamnoides*. The greater cold and freezing tolerance of leaves of male plants than females may be advantageous in terms of cold hardiness of the whole plant. Lennartsson and Ögren [24] suggested that even deciduous trees may benefit from maintaining leaves as long as possible during autumn to allow continued photosynthesis, which may be important in building up reserves needed for cold acclimation. Our observations that male and female *H. rhamnoides* respond differently to a change in environmental conditions display the importance of an unambiguous role of gender for assessing the response of dioecious species to climate change or any other experimental manipulations. Li et al. [25] observed that stressful environments have more negative impact on females and become male-biased in nature as resources become limited.

Plant organs varied in their response to cold stress - typically the leaves are much more sensitive than the shoots. Leaves respond more quickly than shoots when exposed to cold stress. These data clearly suggest that leaves are more sensitive to the environmental cues triggering acclimation than the shoot tissues. The result is in consistent with findings of Li et al. [26] where buds and leaves of Silver birch (*Betula pendula*) are found to be more responsive to the environmental cues than the stem tissue.

Altitude of plant origin demonstrated a significant impact on shoot proline content in *H. rhamnoides.* Linear relationship between increased proline contents and increasing altitude was observed. The results suggested that plants from higher altitude possess more effective mechanisms that prevent the frost damage. This result is in consistent with findings of Greer and Robinson [19] who reported that *Leptospermum scoparium* from high altitude origin are less affected by frost than that from low altitude. Similar result is reported in *Salix pentandra* [27].

5. Conclusion

Our results showed sexually different responses of *H. rhamnoides* to environmental cues in natural conditions. Females suffer more from negative effects of cold and freezing than males. Leaves respond more quickly than shoots when exposed to cold stress. We suggest that when *H. rhamnoides* is planted in cold regions, females should be provided with added protective measures to improve their chances for survival.

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Conflict of interest

The authors have no conflict of interest to report.

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