GENDER DIFFERENCES IN ANTIOXIDANT PROPERTIES, PHENOTYPIC PLASTICITY AND FREEZE TOLERANCE IN SEABUCKTHORN (*HIPPOPHAE RHAMNOIDES* L.) ALONG AN ALTITUDINAL GRADIENT IN TRANS-HIMALAYAN LADAKH, INDIA

Thesis submitted in fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

PHUNTSOG DOLKAR

Enrollment No. 126560

Department of Biotechnology

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY
WAKNAGHAT, DISTRICT SOLAN, H.P., INDIA

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DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the Ph.D. thesis entitled **"Gender differences in antioxidant properties, phenotypic plasticity and freeze tolerance in Seabuckthorn (*Hippophae rhamnoides* L.) along an altitudinal gradient in trans-Himalayan Ladakh, India"** submitted at **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work. I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my Ph.D. Thesis.

(Phuntsog Dolkar)
Enrollment No. 126560
Department of Biotechnology & Bioinformatics
Jaypee University of Information Technology Waknaghat, Solan, India
Date: May 2018

SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled **"Gender differences in antioxidant properties, phenotypic plasticity and freeze tolerance in Seabuckthorn (*Hippophae rhamnoides* L.) along an altitudinal gradient in trans-Himalayan Ladakh, India"** at **Jaypee University of Information Technology, Waknaghat, India,** is a bonafide record of her original work carried out under our supervision. This work has not been submitted elsewhere for any other degree or diploma.

(Dr. Tsering Stobdan)
Defence Institute of High Altitude Research
Defence Research and Development Organisation
Leh, Ladakh-194101
Date: May 2018

(Dr. Anil Kant)
Department of Biotechnology & Bioinformatics
Jaypee University of Information Technology
Waknaghat, Solan- 173215
Date: May 2018

DEDICATED TO

MY LATE GRANDFATHER
"MAMAY DAWA NORZANG"

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LIST OF ABBREVATIONS

AMOVA	Analysis of molecular variance
ANOVA	Analysis of variance
CTAB	Cetyltrimethyl ammonium bromide
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl radical
DW	Dry weight
EL	Electrolyte Leakage
FRAP	Ferric reducing antioxidant power
FRP	Fiberglass Reinforced Plastic Greenhouse
GAE	Gallic acid equivalents
GD	Genetic diversity
GPS	Global Positioning System
IBA	Indole Butyric Acid
IC_{50}	Inhibitory concentration 50
LA	Leaf area
LL	Leaf Length
LT	Leaf thickness
LW	Leaf Width
MCMC	Markov Chain Monte Carlo
MRA	Multiple Regression Analysis
NJ	Neighbor joining
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction

PPM	Parts per million
R&D	Research and Development
R^2	Regression coefficient
SBT	Seabuckthorn
SLA	Specific leaf area
SSR	Simple Sequence Repeats
TAC	Total antioxidant content
TPC	Total phenolic content
TPTZ	2,4,6-tripyridyl-*s*-triazine
UPGMA	Unweighed Pair Group Method with Arithmetic Mean

CHAPTER 1
INTRODUCTION

1.1 Seabuckthorn in trans-Himalaya

Seabuckthorn (*Hippophae rhamnoides* L., Elaeagnaceae) is an ecologically and economically important thorny dioecious shrub native to Asia and Europe. The plant is naturally distributed from sea level in European countries to high altitude in trans-Himalaya, which indicate its wide adaptation to diverse environmental conditions [1], [2]. All plant parts are considered to be reservoir of an array of important health-promoting substances. Female plant produces berries that are rich in vitamins, fatty acids and many other important compounds. Both the gender produces silvery green leaves which are rich in important secondary metabolites. Owing to extensive growth of its root system and the capability to fix atmospheric N_2 (Nitrogen), Seabuckthorn (SBT) is planted to prevent soil erosion and to improve soil fertility thus have an ecological importance. In India it is mainly confined to trans-Himalayan region. Once considered to be a thorny menace, SBT is now being seen as a means for sustainable development of the Himalayan region. Development of SBT-based products has resulted in commercial spin-offs to the extent that demands for SBT from Ladakh region now outstrip the supply. In India SBT received much attention after Defence Institute of High Altitude Research (DIHAR), formerly Field Research Laboratory (FRL), has developed and commercialized the technology for production of beverages from its highly acidic fruit [3]. Important prospective of this health beneficial shrub has been recognized by many Research and Development (R&D) organizations.

Figure 1.1: Male (A) and female (B) plant of Seabuckthorn

1.2 Geographical Distribution

The genus *Hippophae* comprises of seven species. *H. rhamnoides* and *H. tibetana* are found in Ladakh. *H. rhamnoides* is the predominant SBT species in trans-Himalayan Ladakh. The plant grows on a broad range of soil, which ranged from highly acidic soil to soil with high salt deposits [4]. The shrub is mainly distributed in Nubra, Indus and Suru valleys. Natural plantation is mainly distributed along Nubra, Shyok and Indus river. It is also found in Changthang region at an altitude of 15,400 ft amsl [3]. *H. tibetana* is found in selected pockets in Zanskar and Changthang area in the region. Using satellite data, it is estimated that area under pure SBT is 7184 ha while the area under mixed SBT is 2083 ha in Ladakh [5]. In Nubra valley the shrub is estimated to be growing in 2876 ha. Ladakh remains the major site for natural SBT resource with over 70% of the total area (13,000 ha) under SBT in the country [6]. Besides Ladakh, the genus is also found in Lahul – Spiti region of Himachal Pradesh, Uttrakhand and Arunachal Pradesh.

1.3 Traditional and Economic Importance

People living in trans-Himalayan region developed mastery in judicious use of SBT resources. Every part of the plant is traditionally used for an array of purposes such as nutritional supplement, medicine, fencing, tree guard, wind break, firewood, building construction, religious rites, agricultural implements etc. Growing of SBT for fencing in the edges of agricultural fields is considered to improve fertility of soil. In Nubra valley, soil brought from densely growing SBT areas is frequently added to low fertile fields to improve soil fertility. *H. rhamnoides* is recorded as *Bar-sTar* in Tibetan medicine. There are over a hundreds of well-known SBT-based formulations in different pharmacopoeias of *Sowa Rigpa* (Tibetan medicine), which is being practiced in Ladakh [7]. However, the age old information of its usage is gradually declining because of opening of the area to the contemporary world along with availability of alternatives from other parts of the world.

Once considered to be a thorny menace, SBT is now being seen as a means for sustainable development by the people of the trans-Himalayan region. DIHAR has developed several antioxidant rich products from fruit and leaf of SBT to combat high altitude related oxidative stress. Commercialization of the products since the year 2001 has resulted in huge demand for SBT. However, limited natural resource of SBT remains a major limiting factor in product commercialization.

SBT berry collection has become an important income generating activity in the region since year 2001. The mean annual berry harvest from 2004 to 2015 was 213.4±83.9 MT, which is less than 5% of the total available SBT resource in the region. Berry harvesting is done for a short period of 20-30 days in September. In year 2013, 0.8% of the total population of Leh district have directly benefitted from berry collection. Average collection per collector was 254.8 kg resulting in a net income of Rs 8,154 per person in 5-10 days. However, the average income generation of individuals who devoted an average of 12.6 days in the season was Rs 18,375 per head. Majority of the berry collectors are from the needy section of the society and women constitute 67.4% of the work force [8].

Plantation of SBT on barren land by Forest Department and local community for greening and income generation is slowly gaining momentum. The vast barren land in the region can be brought under SBT plantation either by planting along existing water sources or through lifting of water for irrigation from the river. According to an estimate of the Forest Department, 2500 ha of barren land can be brought under SBT plantation without much investment in Leh district. If cultivation is done on 2500 ha in a planned manner the projected net income from SBT alone is estimated to be USD 72 million in the year 2030 [9]. Income generation will increase many-fold if value added products are also manufactured in the region.

1.4 Vegetative Propagation

Vegetative propagation is commonly used method for propagation of woody plants as it is easy to handle and one of the most inexpensive methods. Interest in mass propagation of SBT is rising due to increasing commercial demand for the berry. Hardwood stem cuttings of pencil thickness (5-10 mm diameter) is used for propagation of SBT, which resulted in 46.5-90% rooting success [10], [11], [12]. Other methods such as micropropagation, suckers and seed propagation are also used [13], [4]. Micropropagation of SBT has been standardized in Murashige & Skoog media. For shoot proliferation 0.01 ppm IBA is found optimum while 2.0 ppm BA and 1.0 ppm NAA is optimum for rooting [14]. The standardized method of propagation employed so far are inadequate to meet the increasing demand for nursery plants due to low rooting success, lesser number of cuttings that can be raised from each mother plant and farmer's unfriendly method in case of micropropagation. Besides, the growth of nursery plants in trans-Himalayan region is slow due to cold climatic condition and high water requirement.

In order to meet the commercial demand, a comprehensive study on SBT propagation is needed.

1.5 Bioactive Content

Berry of SBT growing in trans-Himalaya is found to have high concentration of vitamins that includes vitamin C, vitamin A, Riboflavin, Niacin, Pantothenic acid, vitamin B-6 and vitamin B-2. It is rich in minerals which include potassium, calcium, iron, magnesium, phosphorous, sodium, zinc, copper, manganese and selenium [15]. The leaves are source of important secondary metabolites. Korekar et al. [16] used various solvents (water, methanol, acidic 50% methanol, 70% acetone, acidic 50% methanol followed by 70% acetone) to study their extraction effects on the total phenolic content (TPC) and antioxidant capacity of fruit pulp, seeds, leaves and stem bark of SBT. They reported that TPC ranged from 1666-13769 mg GAE/100g DW and observed highest value in seeds followed by stem bark, leaves and pulp. In their study, the FRAP value ranged from 12.61 to 16.83 mM $FeSO_4$ and the highest value was observed in stem bark followed by seeds, leaves and pulp. Besides, the free radical scavenging activity in terms of IC_{50} value was highest in pulp (3.39 mg ml^{-1}) which is 7.8 times higher as compared to that of stem bark (0.43 mg ml^{-1}) and around 2.4 times higher as compared to that of seeds (1.4 mg ml^{-1}). Along with important secondary metabolites, SBT juice contains fatty acids such as palmitoleic, palmitic, oleic, linoleic and linolenic acids and carotenoids [17]. The rare palmitoleic acid proportion in pulp of *H. rhamnoides* ranged from 2350-2650 mg/kg while that of *H. salicifolia* is 692-840 mg/kg.

A comprehensive study on bioactive compounds of SBT in relation to gender is still obscure. Górnaś et al. [18] studied lipophilic antioxidants in mixed SBT samples of 10 females and 10 males. It was found that lipophilic antioxidant was lower in male as compared to male leaves. In contrast, Šnē et al. [19] reported higher total phenolics and antioxidant in female SBT leaves. The antioxidant compounds viz. α-tocopherol, β-tocopherol, γ-tocopherol, plastochromanol-8 and β carotene were observed to be lower in male as compared to female SBT leaves [18].

1.6 Adaptation to Changing Climate

Competition for the availability natural resources are largely affected by change in climate and are likely to hinder the biological systems in terms of their distribution and

abundance [20], [21]. Plants that are sessile and long lived in nature provide a functional experimental field to study the capability of plant to compete and facilitate in order to provide responses to change in climate [22], [23], [24], [25]. The plants that harbors in mountainous ecosystems experience abrupt alteration in abiotic factors that occur in a short distance, which promotes significant changes in the selection pressures on plant life history traits [26]. The variability in environmental factors renders change in phenotype of plants which is commonly known as phenotypic plasticity and a long term selection by these plants can result in development of adaptations in morphological and physiological characters to the confined environment, thus creating an ecotypic differentiation in functional traits [21], [27]. Generally leaves reduce length, width, area and become thicker with increasing altitude [28], [29], [30]. However, the problem in deciphering the well accepted correlation between altitude and leaf morphology is the confounding of environmental and genetic factors [29]. Evidences support that plant originating from different altitudes remains diverse when planted and grown at same elevation [31], [32], [29]. Cordell et al. [33] reported that leaf morphology is mostly determined by genetic factors but leaf anatomical and physiological changes are determined by environmental factors in tree species *Metrosideros polymorpha.* Hovenden and Schoor [29] found that the morphological response to the environment generally overrides the genetic influence in *Nothofagus cunninghamii*. A similar study revealed that extensive distribution of *Pennisetum setaceum* along altitude is the consequence of ecological tolerance rather than adaptation of specific ecotype [34].

The dioecious species may be particularly susceptible against climate change because they often reveal spatial segregation of male and female plants due to exhibition of gender-specific physiological and morphological responses to different microhabitats [35]. Cost of reproduction leads to prioritization of plant resources in reproductive development rather than vegetative growth or any protection in females. A major prioritization towards reproduction is generally associated with the drawbacks in terms of oxidative stress and cellular injuries, especially under antagonistic conditions [36], [37]. It is observed that generally females are more abundant in high-resource micro-sites and males are more common to low-resource micro-sites [38]. Not accounting for sexual variation could lead to incorrect assessment of a species response to climate change [39]. Li et al. [40] reported that at extreme altitude (above 2800 m) the relationship between leaf morphology and altitude differed from the conventional linear relationship along

altitudinal gradients in *Hippophae,* but this aspect has not been studied in details. Being adaptive to vast environmental conditions, SBT may serve as a prime candidate to investigate gender response to climate change.

1.7 Freezing Tolerance

Woody plant species are fixed in soil and are unable to escape the adverse environmental cues as that of animals. To cope with the adverse environmental signal, plants have evolved different adaptive mechanisms during the course of evolution [41], [42]. Among different abiotic stresses, low temperature stress is considered to be one of the common stress that a plants experience constantly during the course of its life cycle. Generally, the temperature from 0-18ºC and below 0ºC is regarded as chilling and freezing temperature, respectively [43]. During the process of growth and development plants sensitive to low temperature cannot survive. In contrast, overwintering plant species, especially those growing in the mountainous regions, respond to environmental cues like low and freezing temperature by a process termed cold acclimation [44], [45]. During cold acclimation plant species undergoes extensive reprogramming in terms of important metabolites and gene expression. However, the mechanisms underlying cold acclimation are not fully understood. Increases in concentration of polyamines, water-soluble carbohydrate [46] or in free amino acids, especially proline [47], [48] are known to be associated with cold acclimation and further acquisition of frost tolerance. Proline is an important low molecular weight amino acid that might act as a compatible solute and help in the protection of membrane proteins against dehydration [49]. It act as molecular chaperones and helps to stabilize membrane integrity or regulation of certain enzymatic systems [50] during stress. There are several lines of indications which indicate that low temperature exposure can alter the structure and the fluidity of cell membranes due to modification of lipids [51].

In dioecious plants gender-related differences in investment towards reproductive biology leads to the differences in morphological and physiological adjustment to cold and freezing conditions. The cost of reproduction involves prioritization of resources in fruit development rather than in vegetative growth or protection in females. In *Poplus cathayana* genders exhibit different adaptive capabilities to a series of abiotic stresses at physiology and proteome level [52], [53], [54], [55], [56]. Zhang et al. [57] reported that male plant contains higher amount of total chlorophyll, soluble sugar and antioxidant

enzyme activities as compared to females *Poplus*. Male exhibits significant higher activity in nitrogen metabolic enzymes and amino acid constituents as compared to female in response to chilling [58].

Furthermore, in nature plant species are subjected to the co-occurrence of multiple stresses. Tolerance to a combination of stresses, predominantly those that faced by plants under natural environmental conditions must be the prime concern of upcoming research works [59], [60]. The majority of environmental stress research works undertaken in the laboratory conditions with controlled elicitors fails to imitate the actual situation that happen in the natural field. A substantial difference might occur between the information that gained artificial stress treatments and the actual knowledge necessary to develop crop plants with improved tolerance to natural field conditions [61], [62]. This might be the reason that some of the transgenic plants which are developed under the laboratory condition with improved tolerance to some biotic or abiotic stress treatments failed to exhibit improved tolerance when taken in the field [63],[64], [65]. However, no studies in SBT have been conducted that addressed the relative importance of the gender for freeze tolerance in natural conditions particularly in the trans-Himalaya.

1.8 Genetic Diversity

Understanding genetic variation and genetic diversity helps to establish conservation and management programme for species and its evolutionary history [66], [67]. Molecular markers played important role in the estimation of genetic diversity in both plant and animal species. Various markers were utilized for diversity characterization in SBT. Korekar et al. [68] studied the genetic diversity of seventeen natural populations of *H. rhamnoides* from major distribution sites of Leh and Nubra valley using AFLP marker along with 20 quantitative morphological characters, and observed significant correlation between the two parameters. They reported that the genetic structure is mainly affected by topography of the region where the mountain ranges acted as natural barrier for gene flow and splits the genetic pool into Leh and Nubra. Their study further suggested moderate genetic diversity in SBT of Ladakh, and Leh population showed higher diversity than that of Nubra valley. Raina et al. [69] used the three Indian species of SBT viz, *H. rhamnoides*, *H. salicifolia* and *H. tibetana* to compare genetic diversity using AFLP and SAMPL and reported that *H. rhamnoides* ssp. *turkestanica* from Ladakh and Lahaul-Spiti are the most diverse and average genetic distance between *H. rhamnoides* from Ladakh

and that of Uttrakhand genotypes is much greater than that obtained among the three species.

In diversity studies, dioecy is considered as one of the basic evolutionary mechanisms that ensure high cross fertilization in trees [70]. Gender-specific genetic diversity study has become essential as it may have a major impact in proper management and genetic improvement. However, so far not much focus has been given to study gender-specific genetic diversity in plants as compared to animals. Zhai et al. [67] reported that although male *Salix* exhibit higher diversity as compared to female but the variation is not significantly different. However, Jia et al. [71] reported that *Myrica rubra* accessions can be divided into male and female clusters. Genetic diversity studies in *Hippophae* are confined to overall diversity and not related to gender difference. Information about the assessment the genetic diversity of male and female populations will aid in getting new genetic combinations [72].

1.9 Selection of High Quality Genotypes

SBT has not received much attention for breeding for high yielding lines with high bioactive contents. There is an increasing demand for high yielding planting material. Berry and leaf harvest in trans-Himalaya are mostly fulfilled from wild populations that vary in quality and quantity of the active ingredient. Approaches for selection of superior genotype are being performed through screening the natural population based on desirable characters including ease of leaf and berry harvest, high fruit yield, less thorny etc. Fulfillment of domestic and industrial demands of crops by harvesting wild resources may reduce the availability of the plants in wild and may gradually lead to destruction of natural resource. Furthermore, for commercial programs systematic crop improvement through the selection of important traits driven by genetic diversity is essential. This could be helpful in better understanding the genetic diversity, quality control, germplasm conservation, and genetic improvement especially where other genetic information like the linkage map is not available [73]. Map-based quantitative trait locus (QTL) analysis is efficient in detecting QTL, but it is time-consuming and labor-intensive [74]. Therefore, concurrent utilization of molecular markers along with morphological and phytochemical attributes to aid breeders in selecting genotypes with desirable traits through marker-assisted selection has proved to be very effective in improving the genetic makeup of crop plants [75] and has been adopted recently in many plants and animals [76], [77], [78].

Marker-trait correlation studies have been conducted in SBT to correlate dried shrink disease [79] and fruit oil quality [80] by using ISSR markers. However, SBT leaves are also gaining importance for being reservoir of significant health promoting compounds and thus marker trait association to reveal the correlation of biochemical data with the molecular marker would be beneficial at early stage of breeding programs.

Thus, considering the above mention problems the present study has been conducted with the following objectives:

1. Development of an improved vegetative propagation method and gender difference in rooting success in *H. rhamnoides.*
2. Gender differences and seasonal variation in total phenolics and antioxidant capacities in *H. rhamnoides* leaves.
3. Gender differences in phenotypic plasticity and adaptive response of *H. rhamnoides* along an altitudinal gradient in trans-Himalaya.
4. Gender-specific seasonal pattern and altitudinal variation in freeze tolerance responses in *H. rhamnoides*.
5. SSR markers based genetic diversity characterization and marker-trait correlation for antioxidant properties in male and female populations of *H. rhamnoides*

CHAPTER 2
DEVELOPMENT OF AN IMPROVED VEGETATIVE PROPAGATION METHOD AND GENDER DIFFERENCE IN ROOTING SUCCESS IN *HIPPOPHAE RHAMNOIDES*

Abstract

Recent interest in the vegetative propagation of SBT arises because of increasing demand for SBT. Vegetative propagation of SBT through pencil thickness (5-10 mm diameter) cuttings is the most preferable method for propagation. However, a more efficient mass propagation system is required for successful plantation of SBT. In this study we made effort to develop an improved vegetative propagation method and study the effect of the plastic mulching, coloured shade netting, spacing, cutting thickness and gender differences on rooting and growth of SBT. Last year growth (one year old) stem cutting of diameter 2.9±0.8 mm thickness resulted in 97.6±2.2% rooting success in greenhouse condition. Use of rooting hormone did not have any significant result. We observed that use of silver black plastic mulching film resulted in 10% higher rooting success and significant plant growth of pencil thickness cuttings in open field condition. Suppression of weed emergence by the plastic mulch resulted in 75.8% time saving in manual weeding by farm workers. Reduction in light intensity by 66% using green shade net resulted in significant reduction in rooting and growth of nursery plants. Rooting and growth related parameters did not significant differed in three different spacing between cuttings suggesting that cuttings can be planted denser (3"×3") under mulching to get higher number of nursery stock per unit area. Cutting thickness showed significant effect on rooting success. Highest rooting percentage was observed in pencil thickness cuttings (7.5±1.6 mm diameter) followed by cuttings with 2.9±0.8 mm and 11.3±1.7 mm basal diameter in open field condition. Gender did not showed significant difference in percent rootability. However, shooting parameters like shoot length, number of primary root and basal diameter of secondary root was found to be higher in male than that of female. Present study could assist in generation of a vegetative propagation technique in which faster growth, larger number of cuttings can be propagated with high rooting success rate.

2.1 Introduction

Woody plants are often propagated through hardwood cuttings as it is one of the most inexpensive methods for vegetative propagation. In general, they require very less special equipment for rooting and can be easily carry out in nurseries [81]. The conventional method for propagating SBT is done through pencil thickness (5-10 mm diameter) hardwood cutting [1], [82], [10, [11], [12]. The drawbacks in using the conventional method for mass propagation include fewer cuttings from each mother plant and low rooting success. Propagating SBT through micropropagation is another option but the problem is that it requires high-tech amenities and well-trained persons to handle which are deficient in remote locations where SBT can be cultivated. Therefore, one of the objectives of the present study was to develop standardized propagation method from last year (one year) growth cutting of 2.9±0.8 mm diameter, which is approximately half the thickness as compared to that of conventional pencil thickness cuttings of 7-8 mm diameter. Growth of nursery plants is slow in trans-Himalayan region because of cold climatic condition. Therefore, the second objective was to delineate the effect of plastic mulching, coloured shade net, spacing, cutting thickness and gender of the plant on rooting success and growth of SBT in nurseries.

2.2 Materials and Method

Two sets of experiments were conducted, one in greenhouse using last year growth cutting of 2.9±0.8 mm diameter, and second in open field condition using pencil thickness cuttings of 7-8 mm diameter. In the first sets of experiment last year (one year old) growth SBT (*H. rhamnoides*) cuttings from stem were taken on 1st to 7th of April from natural SBT stand. The average length of cuttings was 16.4±2.2 cm and the average diameter of basal end was 2.9±0.8 mm. Following collection, thorns was removed from each cutting and preceded for rooting trial. The study also included effects of fourteen different rooting media, and six different indole-3-butyric acid (IBA) concentration (0, 200, 500, 1000, 2000, 5000 ppm). Before treating with different hormonal concentration, the base of each cutting were cut and soaked in tap water for one day and IBA treatment was applied for 1 min, 24 hr and 48 hr. The cuttings were planted into polybag (depth of 3 cm) that was filled with pre-wetted rooting medium, directly after applying treatment, as indicated in Figure 2.1. The trials were performed in a Fiberglass Reinforced Plastic greenhouse. Due to the dry climate of the region, watering was performed daily to improve the moisture

inside the greenhouse and to avoid drying out. Success in rooting was scored three months after planting, at time of which cuttings were carefully separated from the rooting media manually, inspected visually, and number of rooted cutting, number of root, shoot length, number of leaf were calculated.

In the second sets of experiments, SBT stem cuttings of 7-9 mm diameter and 15.3±1.2 cm long were obtained from female plants on 1st to 7th of April in trans-Himalayan Ladakh region. Thorns on each cutting were removed. The cutting base were cut and soaked in tap water for one day followed by 500 ppm indole-3-butyric acid (IBA)

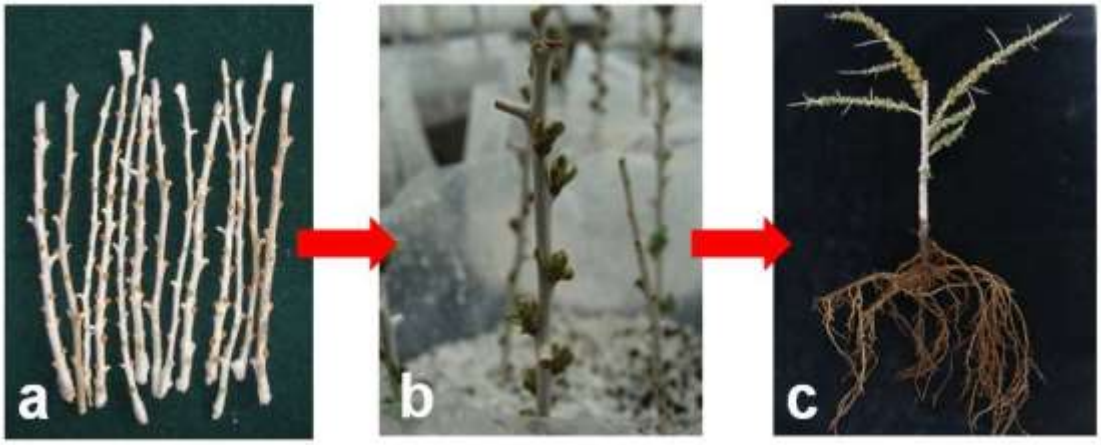


Figure 2.1: Vegetative propagation using last year growth cutting (a: 2.9±0.8 mm diameter cuttings; b: cuttings in rooting media; c: rooted nursery plant)

treatment for 1 min. They were planted in nursery bed at spacing of 6"×6" gap between cutting to cutting and row to row, except in spacing trial. Each plot (4.57 m^2) received 3.5 kg farm yard manure at time of field preparation. Irrigation was provided by flooding system at weekly interval. Silver black plastic film (30 micron) was used for mulching with black surface facing the sun. The result was compared with cuttings under non-mulched condition. The experiment was designed as complete randomized block with three replicates of 126 cuttings each. A similar set of three plots were established in an adjacent section with cuttings from male plant. Weed emergence and time consumed in manual weeding in mulched and non-mulched plots was calculated in terms of fresh weight of weed (kg/plot) and time devoted by a single farm worker in weeding (min/plot), respectively. To study the effect of shading, three replicates of 126 cuttings each were shaded with a green net used in nurseries to give approximately 60% reduction of sunlight. The nets were fixed on quonset-shaped structures consisting of metal pipes framework

with 2'6" height at the centre. Rooting success and plant growth was compared with cuttings grown in open field conditions. The light intensity measured with Datalogging light meter (HD450, Extech Instruments) at noon in open field was 131193±43574 lux and that of shade net conditions was 44353±17335 lux. Therefore, the net used reduced light intensity by 66.2%. Three nursery spacing treatment (6"×6", 3"×3", triangular system i.e additional one cutting in the centre of 6"×6") was studied. The experiment was designed as complete randomized block with three replicates of 126-480 cuttings each depending on spacing. To study the effect of cutting diameter, three cuttings thickness with basal diameter of 11.3±1.7 mm, 7.5±1.7 mm and 2.9±0.8 mm were taken. The experiment was designed as complete randomized block with three cutting thickness with three replicates of 126 cuttings each in open field condition. Rooting success and plant growth parameters were scored in mid-October. Ten rooted cuttings from each replicate were sampled from different treatments. After washing the rooted cuttings, root and shoot growth related parameters were recorded. One-way ANOVA was performed with the help of 2-sided Tukey's HSD at $P \leq 0.05$. All statistical analysis was performed using SPSS for Windows 17.0 version.

2.3 Result and Discussion

Development of an improved vegetative propagation method using last year growth cutting (2.9±0.8 mm diameter) in greenhouse:

Use of last year growth cutting of 2.9±0.8 mm diameter resulted in significantly higher rooting success in greenhouse condition (Fig. 2.1). Different rooting media significantly affect rooting percentage and plant growth (Table 2.1.1). Manure, perlite, soil, sand and peat moss: perlite (1:2) resulted in highest rooting success (>98%). The rooting success was above 94% in all the rooting media which suggests that all media tested are suitable for rooting. The mean rooting percent (97.6±2.2), was significantly higher in comparison to the previous studies using pencil thickness hardwood cutting with 46.5-90% rooting success [10], [11], [12]. Rooting success depends on the thickness of cutting. Exadaktylou et al. [81] reported highest rooting percentage in cherry rootstock in cutting with diameters of 6-8 mm and 9-11 mm, whereas cuttings with diameter 12-14 mm diameter showed no rooting.

Table 2.1.1: Combined effect of different rooting media on rooting percentage and growth parameters of 2.9±0.8 mm diameter cuttings in greenhouse after 13 weeks

Rooting media	Rooting %	Shoot length per cutting (mm)	Number of shoot per cutting	Number of leaf per shoot	Number of primary root per cutting	Number of secondary root per cutting	Length of the longest primary root (mm)	Length of the longest secondary root (mm)	Diameter of basal end of primary root (mm)
M1	93.9±1.5^{a}	58.4±17.0^{bc}	7.6±3.0^{a}	22.7±6.1^{ab}	7.1±3.5^{ab}	69.1±27.7^{abc}	148.1±56.2^{ab}	48.2±16.1^{def}	1.2±0.2^{ab}
M2	98.7±2.3^{b}	60.3±23.9^{bc}	10.4±2.5^{a}	21.6±7.9^{ab}	7.0±2.5^{ab}	85.5±39.5^{bc}	**205.8±58.7^{b}**	**64.0±33.8^{f}**	1.1±0.2^{ab}
M3	97.8±3.2^{ab}	52.2±17.6^{abc}	7.8±3.0^{a}	21.4±7.9^{ab}	6.7±3.1^{ab}	65.9±26.4^{abc}	144.5±69.4^{ab}	47.4±15.9^{cdef}	1.4±1.1^{ab}
M4	97.9±2.0^{ab}	45.3±16.3^{ab}	8.9±2.2^{a}	20.1±3.9^{ab}	5.9±2.5^{a}	71.8±28.8^{abc}	141.0±65.7^{ab}	35.7±16.9^{abcde}	1.0±0.2^{ab}
M5	95.3±4.5^{ab}	49.0±19.2^{abc}	7.9±4.0^{a}	18.2±5.3^{ab}	5.1±2.5^{a}	58.2±23.9^{ab}	142.0±70.5^{ab}	22.9±12.0^{a}	1.0±0.3^{a}
M6	96.1±3.5^{ab}	48.9±16.1^{abc}	7.9±2.8^{a}	18.6±5.3^{ab}	5.1±2.3^{a}	51.3±22.8^{a}	105.7±50.2^{a}	25.7±17.7^{ab}	1.1±0.3^{ab}
M7	97.4±2.1^{ab}	50.3±13.8^{abc}	9.6±3.5^{a}	19.5±4.0^{ab}	5.3±2.1^{a}	42.8±17.0^{a}	116.5±46.6^{a}	30.7±12.1^{abcd}	1.3±0.4^{ab}
M8	97.2±2.2^{ab}	58.1±15.8^{bc}	8.4±3.0^{a}	21.1±3.7^{ab}	6.2±2.2^{ab}	68.0±25.7^{abc}	127.6±64.4^{ab}	26.4±10.0^{ab}	1.2±0.2^{ab}
M9	97.4±2.1^{ab}	**67.9±21.4^{c}**	9.4±2.2^{a}	23.1±7.8^{b}	6.8±2.4^{ab}	65.9±23.3^{abc}	176.8±73.5^{ab}	46.4±25.6^{bcdef}	1.0±0.2^{a}
M10	97.8±2.0^{ab}	65.3±24.2^{bc}	8.6±2.6^{a}	21.3±7.7^{ab}	7.5±3.^{ab}	69.3±31.6^{abc}	187.3±111.4^{ab}	54.9±21.7^{ef}	1.2±0.3^{ab}
M11	98.9±2.1^{b}	37.1±16.4^{a}	8.9±2.8^{a}	17.3±4.6^{ab}	5.4±2.7^{a}	45.1±22.6^{a}	183.4±163.1^{ab}	27.3±12.6^{abc}	1.4±0.4^{ab}
M12	98.9±1.1^{b}	59.4±15.8^{bc}	9.8±2.3^{a}	**23.3±4.6^{b}**	**9.5±4.6^{b}**	74.2±24.2^{abc}	173.2±71.4^{ab}	37.1±13.3^{abcde}	**1.5±0.3^{b}**
M13	**99.8±0.2^{b}**	33.2±7.8^{a}	**10.6±3.3^{a}**	16.5±3.1^{a}	6.1±2.4^{ab}	49.9±15.3^{a}	151.6±45.9^{ab}	30.8±15.7^{abcd}	1.4±0.3^{ab}
M14	99.1±1.0^{b}	57.8±19.5^{bc}	10.4±2.3^{a}	21.2±3.1^{ab}	7.7±4.1^{ab}	**97.4±53.5^{c}**	124.3±68.0^{ab}	35.5±20.4^{abcde}	1.4±0.4^{ab}
Mean	97.6±2.2	53.1±19.9	9.0±3.0	20.4±5.9	6.5±3.1	64.8±32.1	152.0±81.4	38.1±21.5	1.2±0.4

M1: peat moss : perlite (2:1), **M2:** peat moss : perlite (1:2), **M3:** peat moss : vermiculite (1:1), **M4:** peat moss : manure (1:1), **M5:** peat moss : soil (2:1), **M6:** soil : manure (2:1), **M7:** sand : manure (2:1), **M8:** sand : soil (2:1), **M9:** vermiculite, **M10:** peat moss, **M11:** soil, **M12:** sand, **M13:** manure, and **M14:** perlite.

Values represented as mean±SD; for each column, different lowercase letters within each column indicate significantly different at P≤0.05, as measured by 2-sided Tukey's HSD

Soaking duration in different hormone significantly affected the percent rootability. Cuttings that were soaked in hormone for more than 1 min failed to root. Cuttings that were treated for 1 min showed over 95% rooting percentage. Different rooting hormone concentrations significantly affected rooting percentage and number of shoot (Table 2.1.2). However, the differences between treatments for shoot length, number of leaf, number of root and root length were not statistically significant. A maximum of 98.8% rooting at 1000 ppm IBA was observed. It appears, however, that exogenous auxin treatments lack any benefit for rooting in one year old growth SBT cutting. Rooting was over 97% without hormonal treatment. In comparison, the application of exogenous hormone is often required and, sometimes, an effective treatment for promoting roots formation in pencil thickness hardwood cuttings in SBT [1], [82], [10], [11], [12]. Rooting was not observed when cuttings were not subjected to exogenous application of rooting hormone [82], [11].

Effect of mulching, shading, spacing, cutting diameter, and gender on propagation of Seabuckthorn by cuttings:

Black plastic mulching significantly increased rooting percentage and growth of nursery plants (Table 2.2.1). Approximately 10% higher rooting was observed in mulched as compared to non-mulched in open field conditions suggesting that mulching provide a suitable condition for higher rooting success for SBT (Fig. 2.2). However, mulching does not significantly increase number of shoot per cutting. Higher rooting success and growth of nursery plants in mulched condition may be attributed to several factors. In the present study, average monthly soil temperature recorded at noon at 10 cm below soil surface in mulched soil was 1.6-3.6ºC higher than non-mulched soil. Therefore, higher soil temperature particularly in the early growth of the plant is beneficial in cold climatic region such as the trans-Himalayan Ladakh region. Since irrigation intervals were similar for both treatments, it is possible that lower evaporation rates beneath the plastic mulch were responsible for higher growth. Suppression of weeds is yet another important factor contributing to higher plant growth in mulched condition. Mulching significantly reduce the emergence of weed. Fresh weight of weeds recorded in mulched soil was 2.0±0.7 kg/plot as compared to 6.4±2.4 kg/plot in non-mulched soil. Suppression of weed emergence by the plastic mulch resulted in 75.8% time saving in manual weeding by farm workers (mulched: 18.7±2.5 min/plot; non-mulched: 77.3±2.6 min/plot).

Table 2.1.2: Combined effect of rooting hormone (IBA) concentration on rooting percentage and growth parameters of 2.9±0.8 mm diameter cuttings in greenhouse after 13 weeks

IBA treatment (ppm)	Rooting %	Shoot length per cutting (mm)	Number of shoot per cutting	Number of leaf per shoot	Number of primary root per cutting	Number of secondary root per cutting	Length of the longest primary root (mm)	Length of the longest secondary root (mm)	Diameter of basal end of primary root (mm)
0	97.4±2.9^{xy}	48.7±20.2^{x}	9.2±3.2^{xy}	19.7±7.0^{x}	5.9±2.6^{x}	59.9±29.9^{x}	158.1±112.6^{x}	38.9±27.6^{x}	1.1±0.4^{x}
200	96.9±2.5^{xy}	**54.8±18.1^{x}**	8.9±3.5^{xy}	20.8±4.9^{x}	6.4±3.1^{x}	62.1±28.5^{x}	143.1±55.6^{x}	35.9±18.8^{x}	1.2±0.3^{x}
500	95.5±4.4^{x}	54.5±20.8 ^{a}	9.5±2.5^{xy}	20.6±6.6^{x}	6.5±3.2^{x}	67.5±31.2^{x}	**161.0±80.5^{x}**	40.1±22.6^{x}	1.2±0.3^{x}
1000	**98.8±0.9^{y}**	54.4±20.1^{x}	8.0±3.0^{x}	**21.4±5.9^{x}**	6.4±3.8^{x}	66.6±40.5^{x}	160.2±72.6^{x}	**42.4±23.8^{x}**	1.3±0.3^{x}
2000	98.7±1.1^{y}	53.9±18.2 ^{x}	**10.0±2.8^{y}**	21.0±5.0^{x}	6.7±2.4^{x}	66.0±26.1^{x}	159.9±87.0^{x}	36.2±16.5 ^{x}	1.2±0.3^{x}
5000	98.3±1.4^{y}	52.3±22.5^{x}	8.5±2.4^{xy}	19.0±5.4^{x}	**7.3±3.5^{x}**	**69.9±35.1^{x}**	129.6±67.5^{x}	35.0±18.2^{x}	**1.4±0.8^{x}**
Mean±SD	97.6±2.2	53.1±20.0	9.0±3.0	20.4±5.9	6.5±3.1	65.3±32.1	152.0±81.4	38.1±21.5	1.2±0.4

Values represented as mean±SD; for each column, different lowercase letters within each column indicate significantly different at P≤0.05, as measured by 2-sided Tukey's HSD

Mulching is now extensively used for large scale SBT nursery raising (Fig. 2.3). No significant gender difference in rooting success was observed in mulched condition (Table 2.2.1). However, significantly higher shoot length, number of primary root and basal diameter of secondary root was observed in male as compared to female plants. In contrast, Dhyani et al. [83] reported higher rooting success and number of roots per cuttings in female cuttings than in male donor plants in *H. salicifolia*.

Effect of shading on the rooting and growth of SBT cuttings has not been reported earlier. If nurseries can be raised under low light without impairing their rooting success and quality, a considerable amount of water will be saved, which is important in many drought-prone areas where water is a scarce resource. However, in the present study, reduction in light intensity by 66% using green shade net was found to significantly reduce the rooting success, shoot length, number of leaf, length and number of roots in mulched condition (Table 2.2.1) (Fig. 2.2). Study on relationship between spacing and growth of

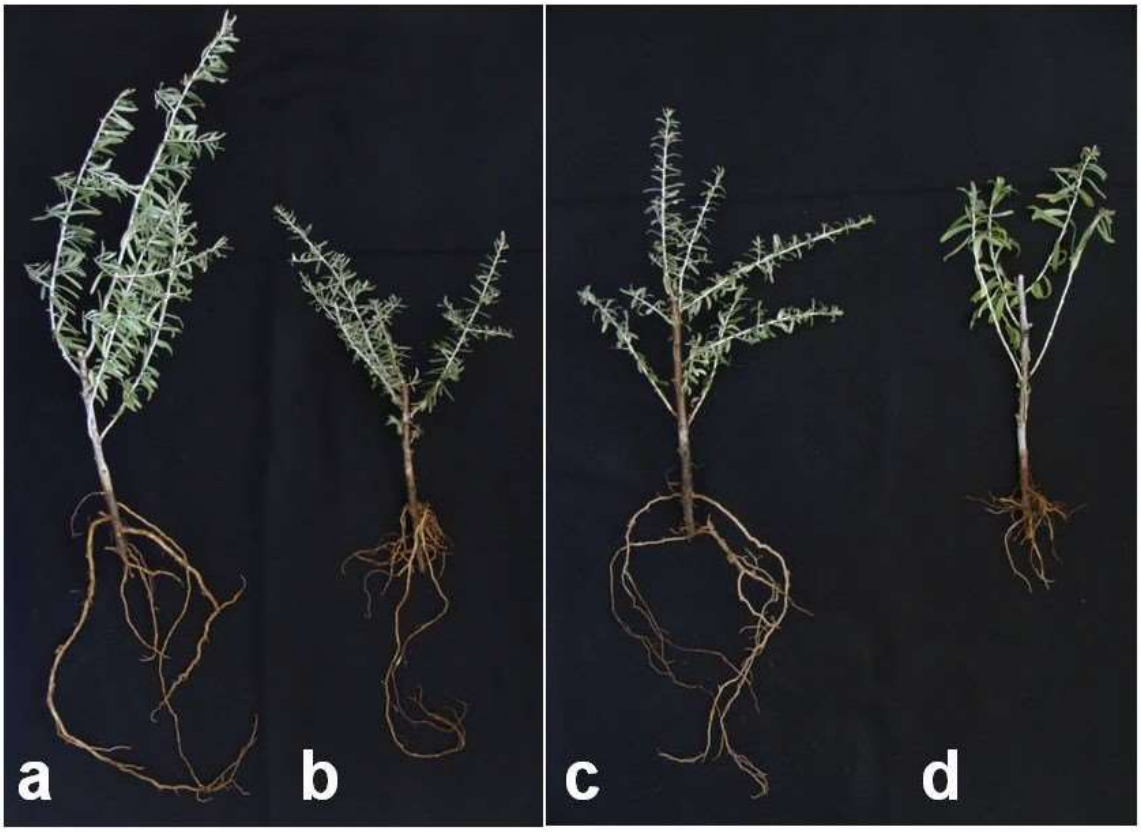


Figure 2.2: Effect of mulching (a: mulched; b: non-mulched) and shading (c: open; d: shade) on growth of Seabuckthorn nursery plant

Figure 2.3: Use of mulching for Seabuckthorn nursery raising in Ladakh

nursery plant is important for nursery managers. If more number of nursery plants can be raised per unit area without affecting the nursery quality, will results in higher income. Results in the present study on three different spacing between cuttings under mulching did not show significant difference in rooting success and growth related parameters in one year old nurseries (Table 2.2.2). Therefore, SBT cuttings can be planted denser (3"×3") to get higher number of nursery stock per unit area. Wider spacing between seedlings is reported to result in higher nutrient uptake and better outplanting performance in two year old nursery stock of Douglas-fir (*Pseudotsuga menziesii*) and Sitka spruce (*Picea sitchensis*) [85].

Cutting diameter showed significant effect on rooting success but did not affect growth related parameters (Table 2.2.3). Highest rooting percentage in open field condition was observed in pencil diameter cuttings (7.5±1.7 mm diameter) followed by

Table 2.2.1: Effect of mulching, shading and gender of donor plant on rooting percentage and growth of Seabuckthorn cuttings (7-8 mm diameter) in open field nursery

Treatment	Rooting %	Number of shoot per cutting	Average shoot length (cm)	Number of leaf per shoot	Number of primary root per cutting	Number of secondary root per cutting	Length of longest primary root (mm)	Length of longest secondary root (mm)	Diameter of basal end of primary root (mm)	Diameter of basal end of secondary root (mm)
Mulch (female)	84.0±5.3^{b}	7.2±2.0^{a}	20.3±3.8^{bc}	40.1±7.3^{c}	8.3±3.3^{ab}	60.8±12.2^{a}	63.3±27.4^{b}	25.8±3.4^{b}	3.5±0.6^{a}	1.6±0.5^{ab}
Non-mulch	75.7±4.1^{ab}	5.7±0.6^{a}	13.5±1.7^{ab}	28.0±6.1^{ab}	6.0±1.7^{ab}	38.3±11.6^{abc}	34.8±3.6^{ab}	14.1±6.6^{ab}	2.2±0.3^{a}	0.8±0.1^{a}
Mulch + shade	78.3±0.6^{ab}	5.8±1.2^{a}	15.6±2.1^{ab}	34.8±6.1^{bc}	7.5±2.1^{ab}	35.0±9.8^{ab}	32.0±18.2^{ab}	10.8±5.4^{a}	2.1±0.6^{a}	0.7±0.3^{a}
Non-mulch + shade	72.7±1.1^{a}	4.0±1.8^{a}	9.3±6.5^{a}	17.7±6.6^{a}	4.0±1.8^{a}	23.5±14.9^{a}	16.7±10.2^{a}	6.4±2.4^{a}	2.2±1.4^{a}	0.8±0.4^{a}
Mulch (male)	84.7±2.5^{b}	5.3±2.2^{a}	28.1±7.5^{c}	42.9±2.4^{c}	9.0±1.3^{b}	55.7±16.3^{bc}	63.1±16.2^{b}	25.2±11.6^{b}	3.5±0.4^{a}	2.1±0.6^{b}

Values represented as mean±SD of 30 cuttings; for each column, different lowercase letters within a column indicate significantly different at P≤0.05, as measured by 2-sided Tukey's HSD

Table 2.2.2: Effect of plant spacing on rooting percentage and growth of Seabuckthorn cuttings (7-8 mm diameter) in open field nursery

Treatment	Rooting %	Number of shoot per cutting	Average shoot length (cm)	Number of leaf per shoot	Number of primary root per cutting	Number of secondary root per cutting	Length of longest primary root (mm)	Length of longest secondary root (mm)	Diameter of basal end of primary root (mm)	Diameter of basal end of second aryroot (mm)
6"×6"	83.5±1.1^{a}	6.7±1.0^{a}	23.9±4.9^{a}	43.0±12.1^{a}	8.2±3.8^{a}	62.5±8.4^{a}	55.6±4.1^{a}	22.7±2.8^{ab}	4.1±0.6^{a}	1.4±0.3^{a}
3"×3"	84.0±5.3^{a}	7.2±2.0^{a}	20.3±3.8^{a}	40.1±7.3^{a}	8.3±3.3^{a}	60.8±12.2^{a}	63.3±27.4^{a}	25.8±3.4^{ab}	3.5±0.6^{a}	1.6±0.5^{a}
*Triangular	84.0±1.2^{a}	5.8±0.8^{a}	19.0±3.1^{a}	40.5±4.8^{a}	7.5±1.8^{a}	46.2±13.2^{a}	66.1±20.2^{a}	12.9±5.4^{a}	3.7±0.4^{a}	1.3±0.5^{a}

Values represented as mean±SD of 30 cuttings; for each column, different lowercase letters within a column indicate significantly different at P≤0.05, as measured by 2-sided Tukey's HSD; *Triangular: additional one cutting in the centre of 6"×6"

cuttings with less than half the pencil diameter (2.9±0.8 mm diameter) and those with more than pencil thickness diameter (11.3±1.7 mm). Therefore, pencil thickness cutting is most suitable for propagation of SBT in open field conditions. Higher rooting percentage in these cuttings is in agreement with studies by Exadaktylou et al. [81] on cherry rootstock. The authors reported that cherry rootstock cutting with diameters of 6-8 mm and 9-11 mm showed the highest rooting percentage, whereas no rooting was observed for those with 12-14 mm diameter.

Table 2.2.3: Effect of cutting thickness on rooting percentage and growth of Seabuckthorn cuttings in open field nursery

Treatment	Rooting %	Number of shoot per cutting	Average shoot length (cm)	Number of leaf per shoot	Number of primary root per cutting	Number of secondary root per cutting	Length of longest primary root (mm)	Length of longest secondary root (mm)	Diameter of basal end of primary root (mm)	Diameter of basal end of secondary root (mm)
> Pencil diameter	50.4±6.6[a]	6.7±2.1[a]	13.2±6.8[a]	27.5±9.5[a]	7.3±3.5[a]	36.3±2.1[a]	50.6±21.3[a]	10.7±3.0[a]	2.2±0.3[a]	0.8±0.2[a]
Pencil diameter	75.7±4.1[ab]	5.7±0.6[a]	13.5±1.6[a]	28.0±6.1[a]	6.0±1.7[a]	38.3±11.6[a]	34.8±3.6[a]	14.1±6.6[a]	2.2±0.3[a]	0.8±0.1[a]
<Pencil diameter	65.0±1.7[ab]	5.3±0.6[a]	12.4±0.5[a]	25.5±2.1[a]	6.3±0.6[a]	33.7±2.1[a]	37.3±1.2[a]	19.8±3.4[a]	2.4±0.4[a]	0.8±0.2[a]

Cutting diameter:> Pencil diameter (11.32±1.7 mm); Pencil diameter (7.45±1.6 mm); <Pencil diameter (2.9±0.8 mm)

Values represented as mean±SD of 30 cuttings; for each column, different lowercase letters within a column in each treatment (A, B and C) indicate significantly different at P≤0.05, as measured by 2-sided Tukey's HSD; *Triangular: additional one cutting in the centre of 6"×6"

2.4 Conclusion

One year old (last year growth) stem cutting of 2.9±0.8 mm thickness, which is comparably less than half the thickness to conventional method of pencil thickness of 7-8 mm hardwood cutting resulted in 97.6±2.2% rooting success in greenhouse condition. Application of exogenous auxin treatments did not have any significant benefit and percent rooting was over 97 without hormonal treatment. Seven-fold more number of cuttings was acquired from each mother plant in comparison to pencil thickness hardwood cuttings, which is the standardized conventional method of SBT propagation. The result obtained from the present study could facilitate in establishment of a vegetative propagation technique wherein larger number of cuttings from each mother plant can be propagated with higher success in rooting rate without the need for application of exogenous rooting hormone.

Use of black plastic mulching resulted in 10% higher rooting success and 75.8% time saving in manual weeding. Reduction in light intensity by 66% using shade net is not advisable for nursery managers since it resulted in significant reduction in rooting success and growth of nursery plants. SBT can be planted denser (3"×3") under mulching condition to get higher number of nursery stock per unit area. Pencil thickness (7.5±1.7 mm diameter) cutting is most suitable for propagation of SBT in open field conditions. However, cutting thickness with 2.9±0.8 mm and 11.3±1.7 mm basal diameter can also be used for propagation with high success rate. The result of the current study could facilitate in establishment of a vegetative propagation technique wherein faster growth and larger number of cuttings can be propagated with high success rate.

CHAPTER 3
GENDER DIFFERENCES AND SEASONAL VARIATION IN TOTAL PHENOLICS AND ANTIOXIDANT CAPACITIES IN *HIPPOPHAE RHAMNOIDES* LEAVES

Abstract

SBT leaves are reservoir of important health promoting bioactive compounds and are used for product development. In this study we made an attempt to delineate the gender differences and harvesting season on total phenolic content (TPC) and total antioxidant capacity (TAC) in SBT leaves. We collected leaf samples that comprised of 200 plants (100 ♂ and 100 ♀) from six natural populations and carried methanolic and acetone extraction for quantification of TPC and TAC. Significantly lower TPC (95.0±23.8 mg GAE/g DW) was observed in females as compared to males (100.8±23.9 mg GAE/g DW). Likewise, significantly lower antioxidant activity in terms of FRAP was detected in females (6.1±1.2 Fe^{2+} mmol/g DW) as compared to males (6.5±1.1 Fe^{2+} mmol/g DW). Significant increase in TPC was observed in male leaves from July to October followed by a significant decrease in November. However, increase in TPC was observed up to August in female leaves and then showed steady declining trend. Similar trend was observed in TAC in both the gender except that female also showed increasing TAC from July to October. October is the best time to harvest SBT leaves, and that leaves contain significantly higher hydrophilic than lipophilic phenolics and antioxidants.

3.1 Introduction

SBT berry and seed have been studied extensively for nutritional and pharmacological properties. However, in recent years SBT leaves are also gaining importance for its nutritional and therapeutic properties. SBT leaves are known to exhibit antimicrobial, anti-viral, anti-radiation, hepatoprotective, cytoprotective, adaptogenic, and immunoprotective activities [86], [87], [88], [89], [90], [91], [92], [93]. These activities are mainly attributed due to high antioxidant activity including phenolics compounds. Antioxidant property of SBT leaves have been studied extensively [90], [91], [94], [16]. Being dioecious species gender differences in bioactive content in different parts of the plant is expected due to different resource allocation by male and female plants. Till date, limited study has been carried out to understand the gender-related differences in TAC and TPC in SBT leaves. Górnaś et al. [18] carried studies on lipophilic antioxidant compounds in mixed sample of 10 males and 10 females SBT. They observed that male contains higher lipophilic antioxidant than that of female leaves. In contrast, Šnē et al. [19] reported higher TPC and TAC in female SBT leaves. Higher antioxidant compounds, such as α-tocopherol, β-tocopherol, γ-tocopherol, plastochromanol-8 and β-carotene were observed in females as compared in male SBT leaves [18]. Contrasting results from limited studies warrant more study with larger number of samples to better understand the sexual differences in TPC and TAC. Besides gender differences, harvesting season affects the antioxidant compound contents in leaves [95], [96], [97], [98]. SBT leaves can be harvested from June to November with a varying ease of harvesting. But till date very few studies have been undertaken to understand the influence of seasonal variation on TPC and TAC in SBT leaves. Morgenstern et al. [99] studied change in TAC and TPC from April to July. Górnaś et al. [18] studied antioxidant activity in mixed samples of female and males from June to October. However, to the best of our knowledge studies on large sample size with an extended harvesting period have not been reported.

3.2 Materials and Methods

3.2.1 Sample collection

A total of 200 SBT shrubs (100 males and 100 females) from six major distribution sites (Fig. 3.1; Table 3.1) were selected for harvesting leaves to study gender-related differences in TPC and TAC. Leaf samples (5g/ plant) were harvested and freeze dried (ALPHA 2-4 LD plus, Fisher Bioblock Scientific, France) and stored for analysis.

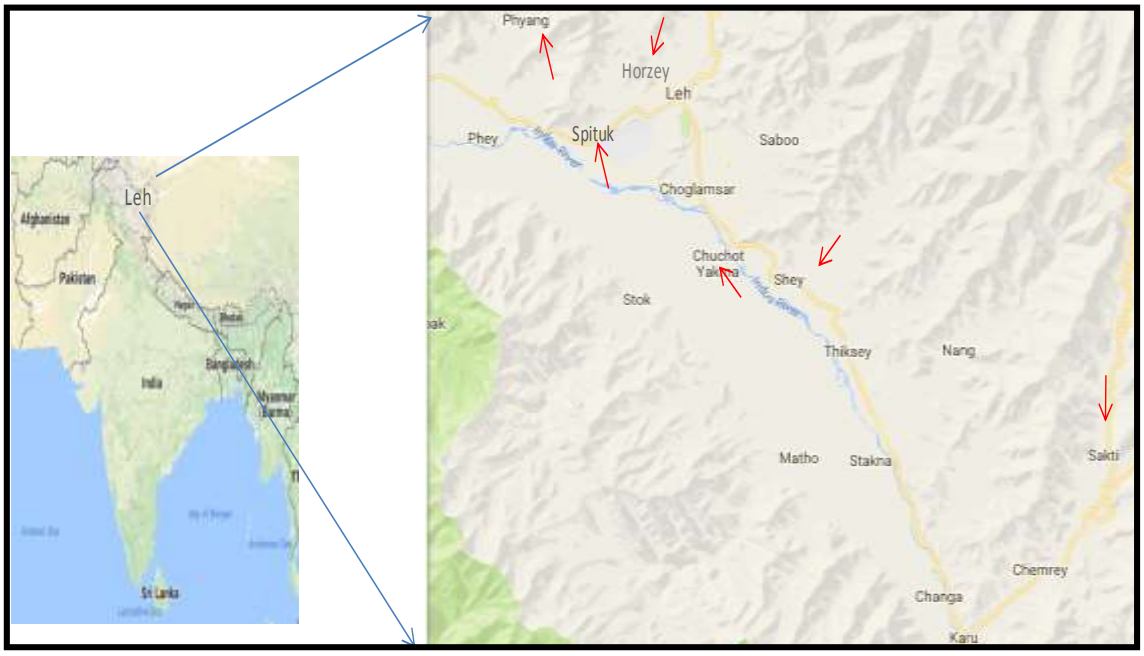


Figure 3.1: Map showing the location of the populations studied (source: Google Maps) (arrow represents sampling site).

Geographical location and altitude of sampling sites are shown in Table 3.1 as established using GPS (GARMIN GPS 72, Olathe, Kansas, USA). Simultaneously, 10 individual plants (5 male and 5 female each) were selected from experimental field of Defence Institute of High Altitude Research (DIHAR) to study the seasonal variation (July to November) in TPC and TAC in leaves. Leaf samples (2 g/ plant) were collected in the first week of every month, freeze dried and stored for analysis.

Table 3.1: Location of *H. rhamnoides* leaf collection sites in trans-Himalayan Ladakh

Sampling Locations	ID	Latitude (N)	Longitude (E)	Altitude (m amsl)	Number of individuals	
					Male	Female
Spituk	SPT	34º07”7’	77º30”4’	3203±5.6	20	20
Chuchot	CHU	34º05”4’	77º35”9’	3239±5.0	17	17
Shey	SHY	34º04”1’	77º37”7’	3260±4.6	17	17
Phyang	PHY	34º11”5’	77º30”1’	3636±49.6	16	16
Horzey	HOR	34º12”1’	77º35”3’	3812±24.8	15	15
Sakti	SKT	34º02”1’	77º48”6’	3885±37.3	15	15

3.2.2 Chemicals

Solvents and Folin-Ciocalteu reagent were obtained from Merck, Germany. 2,4,6-tripyridyl-*s*-triazine (TPTZ), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), gallic acid and ferrous sulfate hexahydrate were from Sigma-Aldrich, USA. All the other chemicals used were of analytical grade.

3.2.3 Extraction

Hydrophilic and lipophilic extraction (two cycle extraction) was performed by the method as described by Korekar et al. [16]. Methanol was used to perform hydrophilic extraction while acetone was used to perform lipophilic extraction. Powdered leaf samples (20 to 40 mg) were mixed with 1.5 ml methanol and vortexed at room temperature for 15 min in a micro centrifuge tube. Following vortex the samples were centrifuged for 10 min at 5600g to obtain the supernatant. The filtrate was mixed with 1.5 ml of acetone and the procedure was repeated as executed for methanol extraction.

3.2.4 Total phenolic content (TPC) assay

TPC was determined following the method by Singleton et al. [100] using Folin-Ciocalteu reagent. An aliquot of the samples (30 µl) was placed into micro-plate (96 well) followed by Folin-Ciocalteu reagent (150 µl), which was previously diluted with distilled water (1:10) and 120 µl sodium carbonate (75 g/l). The micro-plates were vortexed, covered with parafilm and allowed to stand for 30 min. Absorbance at 765 nm was recorded in a micro-plate reader (SpectroMax M2 e, Molecular Devices, Sunnyvale, CA, United States). TPC was expressed in gallic acid equivalents (GAE mg/g DW). The calibration equation for gallic acid was $y=0.014x-0.003$ ($R^2=0.995$) where y is the absorbance at 765 nm and x is the concentration of gallic acid in mg/l.

3.2.5 Ferric reducing antioxidant power (FRAP) assay

FRAP assay was conducted using the method previously described by Ikram et al. [101] with minor modifications [16]. Extract (7.5 µl) and distilled water (22.5 µl) were added to 225 µl of freshly prepared FRAP reagent (10 parts of 300 mmol/l sodium acetate buffer at pH 3.6, one part of 10 mmol/l TPTZ solution and one part of 20 mmol/l $FeCl_3.6H_2O$) and the mixture was incubated for 30 min. Absorbance was measured at 593 nm. The calibration equation for $FeSO_4.7H_2O$ was $y=0.010x-0.011$ ($R^2=0.996$) where y is the absorbance at 593 nm and x is the concentration of $FeSO_4.7H_2O$ in mM. FRAP value

was expressed as mmol Fe (II)/g DW. Free radical scavenging method by DPPH developed by Brand-Williams et al. [102] with minor modification [16]. A 0.1 mmol/l solution of DPPH in methanol was prepared and the solution (300 µl) was treated with pooled methanolic and acetone samples (15 µl). A control was treated with solvent (15 µl) instead of the extract. The mixture was left to stand at room temperature for 30 min before the decrease in absorbance at 517 nm was recorded. Antioxidant value was expressed as IC_{50}, the amount of sample extracted into 1 ml solution necessary to decrease by 50% the initial DPPH concentration. IC_{50} was derived from the % disappearance vs. concentration plot (concentration means mg of SBT leaves on DW basis extracted into 1 ml solution).

3.2.6 Statistical analysis

All the experiments were performed in triplicate. The experimental results were expressed as mean ± standard deviation (SD) using statistical analysis with SPSS (Statistical Program for Social Sciences, SPSS Corporation, Chicago, Illinois, USA). Student's t test and Pearson's correlation analysis were performed to compare and find the correlations among parameters. One way analysis of variance (ANOVA) and post hoc analysis with 2-sided Tukey's HSD at $p \leq 0.05$ level were performed. Regression was performed using MS Excel. Box plots were produced using XLSTAT software.

3.3 Results and Discussion

3.3.1 Effect of plant gender on total polyphenol content and antioxidant activity

High variability in TPC among and within genotypes was observed in different populations. The TPC ranged from 29.9 to 165.8 in females and 47.2 to 173.1 mg GAE/g DW in male genotypes. Therefore, TPC variation of 1-5.5 folds in female and 1-3.7 fold in male was observed. Effect of plant sex on TPC is presented in Table 3.2. Significantly high variability was observed between the populations. Males showed significantly higher TPC than females ($P < 0.001$, Student's t-test) in three out of the six populations. Females showed higher values in two populations and no significant gender difference was observed in the remaining single population. However, the overall mean TPC value of the six population was significantly lower in females (95.0±23.8 mg GAE/g DW) than males (100.8±23.9 mg GAE/g DW) at $p \leq 0.01$. In contrast, Šnē et al. [19] reported

Table 3.2: Gender difference in total phenolic content (mg GAE/g DW) of *H. rhamnoides* (100 males, 100 females) leaves

Population ID	Male leaves					Female leaves				
	Hydrophilic	Lipophilic	Combined[1]			Hydrophilic	Lipophilic	Combined[1]		
		Mean±SD		Min	Max		Mean±SD		Min	Max
SPT	101.60±20.47^{b}	2.27±0.26^{bc}	103.86±20.58^{b}	61.53	142.45	107.76±19.95^{c}	2.27±0.40^{bc}	110.03±20.05^{c}	76.72	165.83
CHU	123.88±21.83^{c***}	2.19±0.22^{ab}	126.07±21.84^{c***}	93.03	173.06	91.67±25.43^{b}	2.95±0.40^{e***}	94.62±25.34^{b}	51.02	152.44
SHY	86.87±18.28^{a***}	2.48±0.40^{c}	89.66±18.90^{a***}	47.19	131.67	74.98±10.95^{a}	2.53±0.49^{d}	77.51±11.15^{a}	51.99	97.03
PHY	93.43±15.18^{ab}	2.03±0.28^{a}	95.46±15.37^{ab}	63.69	124.28	109.59±16.45^{c***}	2.11±0.30^{ab}	111.70±16.70^{c***}	81.86	144.57
HOR	84.71±12.82^{a}	2.49±0.39^{c}	87.21±12.88^{a}	67.04	120.58	94.36±13.65^{b***}	2.38±0.35^{cd}	96.89±14.10^{b***}	73.41	125.42
SKT	99.91±29.63^{b***}	3.12±0.66^{d***}	103.02±29.70^{b***}	58.07	170.36	76.76±26.49^{a}	2.01±0.38^{a}	78.77±26.76^{a}	29.88	132.01
Average	98.24±23.82**	2.41±0.60	100.83±23.92**	47.19	173.06	92.64±23.66	2.37±0.49	95.02±23.82	29.88	165.83

The values are mean ± SD at each sampling point

The lower different letters in lowercase specify that the values differ significantly at $p < 0.05$, measured using 2-sided Tukey's HSD among different populations

[1]Combined: Indicates the combined value of hydrophilic and lipophilic extraction

**value significantly higher as compared to other sex at $p \leq 0.01$; *** value significantly higher as compared to other sex at $p \leq 0.001$

higher total phenolics in female SBT leaves, which could be because of small sample size as observed in PHY and HOR populations in the present study. The overall difference in TPC between male and female SBT leaves in the present study was 5.8%. However, Koczka et al. [103] reported 45% higher TPC in male *Ginkgo biloba* leaves than female.

The ferric reducing activity ranged from 2.6 to 9.1 in female and 3.9 to 9.5 Fe^{2+} mmol/g DW in male. The difference in FRAP value between the genotypes showing the highest and lowest value was 1-3.5 fold in female and 1-2.4 fold in male. Gender effect on FRAP is presented in Table 3.3. High variability was observed between the populations. Males showed significantly higher ferric reducing activity than females ($P< 0.001$, Student's t-test) in two populations and no significant gender differences was observed in the remaining four populations. However, the overall mean FRAP value was significantly lower in females (6.1±1.2 Fe^{2+} mmol/g DW) than males (6.5±1.1 Fe^{2+} mmol/g DW). The result is in agreement with studies by Górnaś et al. [18] who reported lower antioxidant activities in mixed two SBT female than 10 males. In contrast, Šnē et al. [19] reported lower ferric reducing activity in male than female SBT leaves. The antioxidant compounds viz. plastochromanol-8, β-carotene, α tocopherol, β-tocopherol and γ-tocopherol were observed lower in male than in female SBT leaves [18].

Free radical scavenging activity of leaves extract expressed as IC_{50} is presented in Table 3.3. A single population showed significantly higher scavenging activities in males than females ($P< 0.001$, Student's t-test) and no significant gender difference was observed in the remaining five populations. The overall mean scavenging value was higher in males but the difference was not statistically significant. Higher TPC and TAC with acetone suggests that SBT leaves contains significantly lower lipophilic than hydrophilic antioxidants. Lower TPC and TAC in female leaves in the present study is in agreement with the fact that in dioecious species 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 [36].

Table 3.3: Gender difference in total antioxidant capacity of *H. rhamnoides* (100 males, 100 females) leaves

Population ID	Male leaves				Female leaves			
	[1]FRAP ($FeSO_4.7H_2O$ mmol/g DW)			[2]IC_{50} (mg/ml)	[1]FRAP ($FeSO_4.7H_2O$ mmol/g DW)			[2]IC_{50} (mg/ml)
	Hydrophilic	Lipophilic	[3]Combined	[4]Combined	Hydrophilic	Lipophilic	[3]Combined	[4]Combined
SPT	7.07±1.17^{c}	0.13±0.01^{a}	7.21±1.18^{c}	0.34±0.10^{a}	7.21±1.04^{c}	0.14±0.02^{ab}	7.35±1.05^{c}	0.32±0.18^{a}
CHU	7.01±0.61^{c***}	0.14±0.02^{ab}	7.15±0.60^{c***}	0.33±0.03^{a***}	6.05±1.20^{b}	0.18±0.03^{d***}	6.23±1.21^{b}	0.37±0.03^{a}
SHY	5.75±0.71^{ab}	0.15±0.02^{bc}	5.90±0.73^{b}	0.40±0.27^{ab}	5.63±0.62^{ab}	0.17±0.02^{c***}	5.80±0.62^{b}	0.42±0.29^{a}
PHY	6.86±0.63^{c***}	0.16±0.02^{c**}	7.02±0.64^{c***}	0.38±0.05^{ab}	5.54±0.70^{ab}	0.15±0.02^{b}	5.69±0.72^{ab}	0.37±0.03^{a}
HOR	6.09±0.91^{b}	0.15±0.02^{c***}	6.24±0.91^{b}	0.46±0.17^{bc}	6.08±0.82^{b}	0.13±0.02^{a}	6.21±0.83^{b}	0.44±0.13^{a}
SKT	5.28±1.26^{a}	0.15±0.03^{c***}	5.08±1.80^{a}	0.54±0.20^{c}	5.07±1.32^{a}	0.13±0.02^{a}	5.19±1.34^{a}	0.65±0.35^{b}
Average	6.37±1.14***	0.15±0.2	6.51±1.14***	0.41±0.18	5.96±1.19	0.15±0.03	6.11±1.20	0.43±0.23

The values are mean ± SD at each sampling point

The lower different letters in lowercase specify that the values differ significantly at $p < 0.05$, measured using 2-sided Tukey's HSD among different populations

[1]FRAP: Ferric reducing antioxidant power

[2]IC_{50}: Inhibitory concentration, the amount of sample extracted into 1 ml solution necessary to decrease by 50% the initial DPPH concentration

[3]Combined: Indicates the combined value of hydrophilic and lipophilic extraction

[4]Combined: Indicates the combined value of hydrophilic and lipophilic extraction

[**]value significantly higher as compared to other sex at $p \leq 0.01$; [***] value significantly higher as compared to other sex at $p \leq 0.001$

3.3.2 Effect of harvest season on total polyphenol content and antioxidant activity

Effect of the harvest season on TPC is presented in Table 3.4. TPC varied significantly during the harvesting period. Increase in TPC was observed up to August in female leaves and then showed a steady declining trend. However, significant increase in TPC was observed in male leaves from July (66.75±7.16 mg GAE/g DW) to October (93.25±7.14 mg GAE/g DW) followed by a significant decrease in November (73.90±10.96 mg GAE/100 g DW). Decline in TPC from August onward in female as compare to October in male leaves may be due to higher reproductive efforts by female during the sampling period (July-November), females developing fruits while males not reproducing. Male contained significantly higher TPC than female leaves from August to November ($P< 0.001$, Student's t-test). Similar trend was observed in TAC in both the gender except that female also showed increasing TAC from July to October (Table 3.4). Progression in harvest season from July to October is related linearly to the increase in TPC (R^2=0.937) and FRAP (R^2=0.976) in male leaves (Fig. 3.2). However, in females the trend of increase was not observed in TPC. In comparison, Morgenstern et al. [99] studied the change in antioxidant capacity and phenols during SBT leaves development from April to July. Antioxidant capacity increased in first week of May and then decreased in third week of the month. A steady increase was observed from June onwards. The phenols decreased initially and then increased steadily during the study period. However, changes in antioxidant capacity and phenols were not studied beyond July. Górnaś et al. [18] studied antioxidants in mixed SBT samples of 10 male and two female in June and October. Higher antioxidant was observed in samples collected in autumn than in summer in both female and male leaves. Results obtained in the present study over an extended harvesting period suggested that October is the best time for harvesting SBT leaves. Ercisli et al. [96] also observed similar trend in antioxidant activity of tea leaves harvested at three commercial harvest seasons (May 15, July 15, September 15). Highest antioxidant activity was observed at 2nd harvest. Increase in TPC and TAC from July to October may be linked to accumulation of health promoting compounds during leaf developmental stages. Decline in TPC and TAC in November may be due to the beginning of leaf senescence in the plant.

Table 3.4: Seasonal variation in total phenolic content and total antioxidant capacity of *H. rhamnoides* (5 males, 5 females) leaves

Month	Male			Female		
	[1]TPC	[2]FRAP	[3]IC_{50}	[1]TPC	[2]FRAP	[3]IC_{50}
July	66.75 ± 7.15^{a}	$4.12\pm0.20^{a***}$	0.52 ± 0.20^{c}	59.97 ± 16.63^{abc}	3.39 ± 0.50^{a}	0.69 ± 0.57^{a}
August	$81.59\pm10.71^{bc**}$	$5.09\pm0.49^{b***}$	$0.26\pm0.09^{b***}$	69.57 ± 13.04^{c}	3.78 ± 0.37^{ab}	0.52 ± 0.16^{a}
September	$87.38\pm6.13^{cd***}$	$5.62\pm0.51^{bc***}$	$0.19\pm0.08^{ab***}$	63.45 ± 8.02^{bc}	4.27 ± 0.32^{c}	0.73 ± 0.16^{a}
October	$93.25\pm7.14^{d***}$	$6.74\pm0.57^{d***}$	$0.12\pm0.04^{a***}$	55.02 ± 8.04^{ab}	4.98 ± 0.33^{d}	0.85 ± 0.63^{a}
November	$73.90\pm10.97^{ab***}$	$5.68\pm0.85^{c***}$	$0.31\pm0.18^{b***}$	51.47 ± 6.60^{a}	4.09 ± 0.47^{bc}	0.94 ± 0.43^{a}
Average	$80.57\pm12.68^{***}$	$5.45\pm1.02^{***}$	$0.28\pm0.19^{***}$	59.89 ± 12.56	4.10 ± 0.66	0.75 ± 0.45

The values are mean ± SD at each sampling point

The lower different letters in lowercase specify that the values differ significantly at $p < 0.05$, measured using 2-sided Tukey's HSD among different months

[1]TPC: Total phenolic content (mg GAE/g DW)

[2]FRAP: Ferric reducing antioxidant potential ($FeSO_4.7H_2O$ mmol/g DW)

[3]IC_{50}: Inhibitory concentration, the amount of sample extracted into 1 ml solution necessary to decrease by 50% the initial DPPH concentration

[**]value significantly higher as compared to other sex at $p \leq 0.01$; [***] value significantly higher as compared to other sex at $p \leq 0.001$

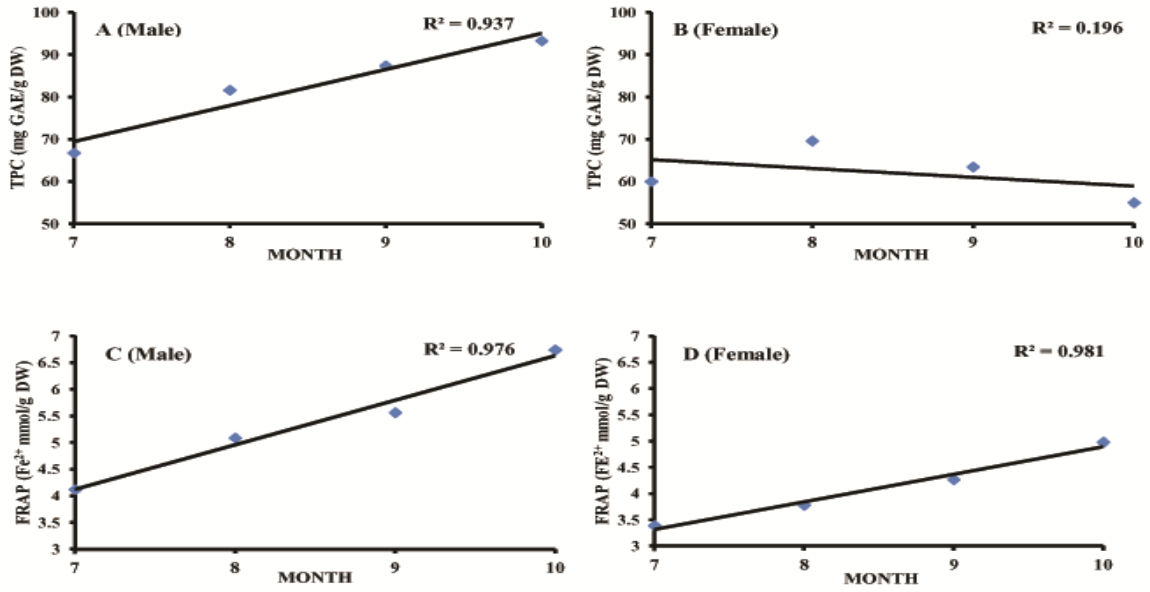


Figure 3.2: Relation between total phenolic content (A-B) and antioxidant capacity (C-D) in male and female SBT leaves with harvest season (July October).

3.3.3 Correlation analysis

A correlation study between TPC and TAC is displayed in Table 3.5. Our data revealed a significant correlation between TPC of male, female and combined samples with FRAP (R=0.423, 0.717, 0.581, respectively) and DPPH (R= -0.208, -0.551, -0.399, respectively) at ≤ 0.01. Similar findings were also observed in SBT berry [104] collected from trans-Himalayan region. Similarly correlation between DPPH and FRAP of male, female and combined samples also showed significant correlation (-0.48, -0.577, -0.533, respectively) at ≤ 0.01.

Table 3.5: Pearson correlation to estimate the interrelationship between TPC, IC_{50}, and FRAP

	Male			Female			Combined		
	[1]TPC	[2]FRAP	[3]IC_{50}	[1]TPC	[2]FRAP	[3]IC_{50}	[1]TPC	[2]FRAP	[3]IC_{50}
[1]TPC	1	.423**	-.208**	1	.717**	-.551**	1	.581**	-.399**
[2]FRAP		1	-.481**		1	-.577**		1	-.533**
[3]IC_{50}			1			1			1

**Correlation is significant at $p \leq 0.01$

[1]TPC: Total phenolic content (mg GAE/g DW)

[2]FRAP: Ferric reducing antioxidant potential ($FeSO_4.7H_2O$ mmol/g DW)

[3]IC_{50}: Inhibitory concentration, the quantity of sample extracted into 1 ml solution necessary to decrease by 50% the initial DPPH concentration

3.4 Conclusion

The presented study showed gender differences and seasonal changes in TPC and TAC in SBT leaves. Significantly lower TPC and TAC were recorded in females as compared to males. Harvesting season has significant influence on TPC and TAC in SBT leaves. The result suggested that October is the best time for harvesting SBT leaves, and leaves contains significantly higher hydrophilic than lipophilic phenolics and antioxidants. Results obtained in this study can be considered for harvesting of SBT leaves for product development and extraction of health promoting compounds.

CHAPTER 4
GENDER DIFFERENCES IN PHENOTYPIC PLASTICITY AND ADAPTIVE RESPONSE OF *HIPPOPHAE RHAMNOIDES* ALONG AN ALTITUDINAL GRADIENT IN TRANS-HIMALAYA

Abstract

We measured leaf morphological characters in male and female *H. rhamnoides* individuals along an altitudinal gradient (2797-4117 m) in trans-Himalayan Ladakh region. Leaves become smaller in length and area, but became thicker with decreasing specific leaf area (SLA) with increasing altitude in both the gender. Leaf size, area, thickness, chlorophyll and petiole length were found to be higher in males than in females, while female had a higher SLA. When cuttings from the plants were grown in a common-garden experiment, the altitudinal effect disappeared for all morphological variables other than leaf thickness and SLA suggesting that most leaf morphological variation in *H. rhamnoides* is environmentally determined, but SLA and leaf thickness are also dependent on genotype. In the event of climate change, our study showed that phenotypic plasticity would be a crucial determinant of plant response in mountainous region. Effect of altitudinal gradient on leaf morphology was more conspicuous in males suggesting that males are more responsive to change in environmental conditions. Stressful environments will cause an added detrimental impact on females than on males. The results suggested that males will adapt better to the changing climate and may lead to a male-biased population in the event of climate change.

4.1 Introduction

Climate change is altering the availability of resources and the conditions that are crucial to plant performance [21]. Over the last few decades, attention is being increasingly focused on evolutionary responses to rapid climate change [26]. One major concern in this context relates to the ability of long-lived species to cope with rapid change in climate [22], [23], [24]. Plant species can adjust to changing climate through environmentally induced shift in phenotype (phenotypic plasticity), adapt through natural selection (genetic response) or migrate to follow conditions to which they are adapted; these options are not mutually exclusive [21]. However, studies of climate change-induced evolution under simulated and natural climatic conditions have rarely integrated plastic and genetic evolutionary responses [105]. In dioecious plants, morphological adjustment to climate change may differ between male and female individuals due to greater reproductive effort in 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 [36]. Not accounting for sexual variation could lead to incorrect assessment of a species response to climate change [39]. However, this aspect has not been studied in detail, particularly in the fragile trans-Himalayan region.

Both abrupt and gradual climate changes will impose selection pressure on plant population [21]. Abrupt climate changes will result in rapid harsh selection for more stress-tolerant genotypes, whereas gradual climate changes are expected to impose soft selection mediated by intraspecific interactions [105]. Altitudinal gradients in stressful mountain ecosystem provide an ideal experimental opportunity for studying the functional traits of plants in response to abrupt climate change. In mountainous regions, sharp changes in abiotic factors occur over short distances, leading to major changes in the selection pressures acting on plant life history traits [26].

Altitude has a major effect on leaf morphology and physiology within a species. Leaves generally decrease in length, width and area but become thicker with increasing altitude [28], [29], [30]. However, the problem in interpreting the well documented relationship between altitude and leaf morphology is the confounding of environmental and genetic factors [29]. There are evidences that plant originating from different altitudes

remain different when grown at same altitude [31], [32], [29]. Cordell et al. [33] reported that leaf morphology is largely genetically determined but leaf anatomy and physiology are environmentally determined in tree species *Metrosideros polymorpha.* Hovenden and Schoor [29] found that the morphological response to the environment generally overrides the genetic influence in *Nothofagus cunninghamii*. A similar study showed that the extensive altitudinal distribution of *Pennisetum setaceum* is the result of ecological tolerance rather than adaptation of specific ecotype [34]. At extreme altitude (above 2800 m) the relationship between leaf morphology and altitude differed from the conventional linear relationship along altitudinal gradients [40], but this aspect has not been studied in details. Thus it appears that the degree of environmental plasticity and adaptation is species and environment dependent.

H. rhamnoides is found in a large altitudinal range, from the sea shores in Europe to over 4694 m in trans-Himalayan Ladakh. Leaf morphological characters including leaf size, thickness and specific leaf area (SLA) are strongly influenced by altitude and gender in this species [40]. However, leaf morphological traits of *H. rhamnoides* measured in natural conditions have 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, genetic and gender factors on relationship between leaf morphology and altitude. The aim of this study was, therefore, to assess how important gender-specific responses are to *H. rhamnoides* in rapidly changing trans-Himalayan environments. This was done using a 'common-garden' experiment, in which large number of cuttings from several male and female shrubs at each of four altitudinal range was grown in a single experimental plot.

4.2 Material and Methods

4.2.1 Study location

The study for this experiment was conducted in trans-Himalayan Ladakh region. The altitude of origin of field-grown plants ranged from 2797-4117 m amsl (Table 4.1). Common-garden experiment was carried out at an experimental farm (34°08.2'N; 77°34.3'E, elevation 3350 amsl) on a flat site with direct sunshine at Defence Institute of High Altitude Research (DIHAR) in trans-Himalayan Ladakh, India. The mean maximum

and minimum temperature during experimental period i.e. 2014-2015 at DIHAR was 12.9±8.8°C and –0.2±9.0°C, respectively. The monthly maximum temperature was highest in July (25.6°C), and the minimum temperature was recorded lowest in January (-13.2°C). The average maximum and minimum humidity was 31.0±4.3% and 24.7±3.7%, respectively. The mean annual precipitation was 163 mm.

Table 4.1: Geographical location and sampling sites of *H. rhamnoides* in trans-Himalaya Ladakh

Altitude ranges (m amsl)	Population	Altitude (m amsl)	No. of samples	
			Male	Female
2800-3000	Turtuk	2797	2	2
	Hundar	2890	1	1
	Diskit	2910	1	1
	Nurla	2990	1	1
3001-3300	Phey	3179	1	1
	Panamik	3180	1	1
	Shey	3232	2	2
	Achinathang	3255	1	1
3500-3800	Shayok I	3576	1	1
	Chemday	3680	1	1
	Shayok II	3740	1	1
	Durbuk	3850	1	1
3801-4200	Horzey	3885	2	2
	Sakti	3927	1	1
	Khardong	4117	1	1

4.2.2 Leaf analysis

Between 13 and 25 August 2014, a single branch was collected from each of 8-10 adult *H. rhamnoides* shrubs from four altitudinal range. All branches were collected on the sunny side of the shrub. Samples of young fully expended leaves (10 leaves per plant) were collected from each branch to investigate the field-grown leaf characteristics. Between 04 and 14 April 2015, dormant cuttings of pencil thickness were taken from each shrub and planted at an experimental farm at DIHAR. Samples of fully expended leaves (10 leaves per plant) were collected in September 2015 from each of the rooted plants to record the garden-grown leaf characteristics. Both field and garden-grown leaves were evaluated for leaf gross morphology. Leaf length, width, thickness were measured using digital calipers (CD-6"CS, Mitutoya, Japan). Leaf length was measured from the base of petiole to leaf tip, while leaf width was recorded at the maximum width of the blade. Leaf thickness was measured from the central part of the lamina, half-way between midrib and margin. Petiole length was taken by cutting the petiole portion of the leaf from base of the leaf blade, while chlorophyll was measured with Chlorophyll Meter SPAD-502 (Konica Minolta Sensing Inc., Japan). Leaf area was measured with a portable leaf area meter (CI 202) (CID Inc, Camas, WA, USA). Leaves were shade-dried to constant mass at 60°C. SLA was calculated by dividing one-sided fresh leaf area by the dry mass.

4.2.3 Statistical analysis

Assumptions of normality were checked for all variables with Kolmogorov-Smirnov test and variables that significantly deviated from normality were log transformed. Tukey's HSD test was performed at $p \leq 0.05$ level for mean comparison. One-way analysis of variance (ANOVA) and regression was conducted with altitude as the fixed factor and leaf morphological parameters as dependent variable. A two-way ANOVA was used to test the relationship of gender, altitude and their interaction with leaf morphological characters. Coefficient of variation (CV) for each trait as a complementary index to interpret the plasticity was computed using the formula: CV = standard deviation × 100/mean. Statistical analysis was carried out in MS excel 2007 and SPSS software package v.17.0 for Windows (SPSS Inc. released in 2008).

4.3 Results and Discussion

4.3.1 Gender differences in phenotypic variation along altitudinal gradient

Both altitude and gender had a significant impact on selected leaf morphology of field-grown *H. rhamnoides* (Table 4. 2). Leaves become smaller in length and area, but became thicker with decreasing SLA with increasing altitude in both the gender. Effect of altitudinal gradient on leaf thickness was more evident in male. The linear regression showed almost linear (R^2 = 0.82) relation between leaf thickness with altitude in male, but there was no linear relationship in female (R^2 = 0.02) (Table 4.3). Petiole length in males increased significantly with increasing altitude (R^2 = 0.44), but similar pattern was not observed in females (R^2 = 0.04). No significant relationship with altitude was observed for leaf width in both the gender. The chlorophyll contents varied between 'high' and 'low' altitudes, with leaves from higher altitude (3500-4200 m) having more than those from lower altitude (2800-3300 m) in both the gender.

Within each altitude, there was a significant influence of gender on selected morphological characters measured. Leaf length, width, thickness, area and chlorophyll contents were higher in males than in females (Table 4.2). The effect of altitude and gender on leaf morphology was supported by results of two-way ANOVA (Table 4.4). Predominant effects of altitude on leaf length (F_1 = 3.8, $p \leq 0.05$), thickness (F_1 = 3.8, $p \leq 0.05$), petiole length (F_1 = 3.3, $p \leq 0.05$), chlorophyll contents (F_1 = 3.0, $p \leq 0.05$), and SLA (F_1 = 12.0, $p \leq 0.001$) was observed. Similarly, significant effect of gender was observed on leaf width (F_1 = 7.0, $p \leq 0.05$), thickness (F_1 = 4.4, $p \leq 0.05$), area (F_1 = 7.5, $p \leq 0.05$) and SLA (F_1 = 7.3, $p \leq 0.01$). Males showed more variability than females in all the leaf morphological traits except for leaf thickness (Table 4.5).

4.3.2 Genetic differentiation in the common-garden experiment

Altitude of plant origin did not have significant impact on leaf morphology of garden-grown *H. rhamnoides* (Table 4.2). No increasing or decreasing trend was observed in leaf length, width, thickness and petiole length with increasing altitude of origin in both the gender. However, a relationship between leaf chlorophyll contents and altitude was observed (R^2 = 0.37) in males, with lower chlorophyll contents in plants from higher altitude origin. Similar relationship was observed between altitude and SLA of garden-grown male plants (R^2 = 0.26), with decreasing SLA with increasing altitude of origin

Table 4. 2. One way ANOVA in filed grown and common garden grown plants and their difference in different sexes.

Gender	Growing condition	Altitude (m amsl)	LL	LW	LT	LA	PL	CC	SLA
Male	Field	2800-3000	35.60±7.85^{d}	3.75±0.32^{a}	0.26±0.01^{ab}	1.41±0.30^{d}	1.66±0.12^{a}	58.35±5.46^{bcdef}	0.21±0.05^{ab}
		3001-3300	30.65±6.62^{abcd}	4.37±0.73^{a}	0.28±0.03^{abcd}	1.28±0.40^{ab}	2.10±0.69^{ab}	62.15±11.78^{cdef}	0.13±0.04^{ab}
		3500-3800	31.73±3.49^{abcd}	4.31±1.23^{a}	0.36±0.03^{bc}	1.27±0.40^{abcd}	2.23±0.1^{ab}	72.59±6.76^{f}	0.14±0.06^{ab}
		3801-4200	28.99±5.09^{abcd}	3.68±0.57^{a}	0.37±0.03^{c}	1.14±0.26^{abcd}	2.64±0.28^{b}	71.74±4.86^{ef}	0.11±0.03^{a}
	Garden	2800-3000	32.52±5.95^{bcd}	3.58±0.86^{a}	0.28±0.05^{abc}	1.12±0.37^{abcd}	1.65±0.38^{a}	63.47±6.21^{cdef}	0.15±0.03^{ab}
		3001-3300	26.08±4.72^{abcd}	3.51±0.75^{a}	0.29±0.07^{abc}	0.86±0.35^{abcd}	1.85±0.52^{ab}	74.79±4.61^{f}	0.11±0.05^{a}
		3500-3800	33.20±5.28^{cd}	3.04±0.37^{a}	0.25±0.09^{a}	0.94±0.16^{abcd}	2.17±0.12^{ab}	69.57±7.42^{def}	0.09±0.03^{a}
		3801-4200	29.07±2.80^{abcd}	3.61±0.25^{a}	0.31±0.04^{abc}	0.96±0.16^{abcd}	2.17±0.24^{ab}	69.36±6.89^{def}	0.09±0.02^{a}
Female	Field	2800-3000	24.79±1.73^{abcd}	3.05±0.31^{a}	0.28±0.04^{abc}	0.75±0.12^{abc}	1.58±0.36^{a}	40.48±1.05^{a}	0.27±0.11^{b}
		3001-3300	23.21±2.23^{abc}	3.58±0.61^{a}	0.26±0.03^{ab}	0.73±0.16^{abc}	1.45±0.16^{a}	50.45±4.30^{abc}	0.26±0.11^{ab}
		3500-3800	21.78±4.16^{ab}	3.34±0.62^{a}	0.29±0.04^{abc}	0.71±0.29^{abcd}	1.41±0.17^{a}	55.16±9.85^{abcde}	0.17±0.02^{ab}
		3801-4200	20.97±4.98^{a}	3.12±0.60^{a}	0.32±0.06^{abc}	0.62±0.21^{ab}	1.46±0.14^{a}	52.70±1.94^{abcd}	0.17±0.05^{ab}
	Garden	2800-3000	21.71±4.64^{ab}	3.43±0.37^{a}	0.28±0.02^{abc}	0.74±0.14^{abc}	1.54±0.15^{a}	41.14±5.99^{ab}	0.23±0.04^{ab}
		3001-3300	21.53±2.23^{ab}	3.28±0.39^{a}	0.28±0.03^{abc}	0.66±0.09^{abc}	1.56±0.35^{a}	59.38±12.19^{cdef}	0.19±0.05^{ab}
		3500-3800	21.47±2.36^{ab}	2.99±0.24^{a}	0.25±0.05^{a}	0.55±0.09^{a}	1.52±0.12^{a}	52.95±2.88^{abcd}	0.21±0.07^{ab}
		3801-4200	21.95±3.65^{abc}	3.33±0.61^{a}	0.29±0.02^{abc}	0.64±0.17^{abc}	1.39±0.10^{a}	54.60±3.61^{abcde}	0.17±0.04^{ab}

LL= leaf length (mm), LW=leaf width (mm), LT=Leaf thickness (mm), PL =Petiole length (mm), LA= Leaf Area ($cm^{2)}$, CC=Chlorophyll contents (SPAD VALUE), DW=Dry Weight (mg), SLA=Specific leaf area (cm^{2}/mg)

Values represented as mean±SD; for each column, different lowercase letters indicate significantly different at P≤0.05, as measured by 2-sided Tukey's HSD between media.

(Table 4.3). However, within each altitude of origin, there was a significant influence of gender on all morphological characters measured. Leaf length, petiole length, leaf area and chlorophyll contents were higher in males than in females (Table 4.2). SLA was significantly higher in females of all altitude of origin except those from 2800-3000 m asl. However, two-way ANOVA results did not support significant effect of gender on leaf morphology in garden-grown plants (Table 4.4). A reduced variability in leaf morphology was observed in garden-grown plant as compared to field-grown plant in both the gender (Table 4.5). However, the exceptions were chlorophyll contents and SLA which remained unchanged in one of the gender. SLA showed opposite trend in field-grown females. The leaves of the low altitude origin (2800-3300 m) garden-grown plants were smaller in length than those collected from the field in both the gender. However, the opposite trend was observed in plants of higher altitude origin (3500-4200 m). Leaf area of garden-grown male plants was smaller than field-grown plants irrespective of their altitude of origin but the values were not significant at high altitudes. However, the trend was not observed in leaves of female plants. Chlorophyll contents of garden-grown leaves remained higher in males of low altitude origin (2800-3300 m) than field-grown plant. However, a lowering trend was observed in leaves of garden-grown plants of higher altitude origin (3500-4200 m). In contrast, no such increasing or decreasing trend was observed in females. The SLA of garden-grown leaves of male plants was lower than those collected from the field. However, in females the trend was observed only in plants of low altitude origin (2800-3300 m). Some of the leaf morphological characters in *H. rhamnoides* are significantly affected by altitude. Leaf size decreased in both the gender with increasing altitude. This trend is consistent with the findings of previous studies [26, 29-30]. Reduction in size is an important strategy employed by plants at high altitude to withstand decrease in temperature and reduced nutrient availability. At high altitude, plants increase supercooling capacity by decreasing cell size and intercellular spaces [106]. Plants decrease the size of their parts to reduce water loss through transpiration, which is a crucial factor in the rain shadowed trans-Himalayan region [30]. Colder soils reduce the water uptake of the root system and induce water stress [107], which might result in reduced size at high altitude. In common-garden experiment, no significant trend was observed in leaf size, suggesting that phenotypic variability along the gradient is due to environmental effect. This trend is consistent with findings of previous studies in other species [26, 29]. In contrast, genetic variation between populations from contrasting environments has

Table 4.3: Regression analysis with altitude as independent variable and field- and garden-grown leaf of *H. rhamnoides* as dependent variable

Parameters	Male				Female				Mixed (male + female)			
	Field-grown		Garden-grown		Field-grown		Garden-grown		Field-grown		Garden-grown	
	R^2	P	R^2	P	R^2	P	R^2	P	R^2	P	R^2	P
Leaf Length (mm)	0.08	0.243	0.55	0.351	0.01	0.840	0.00	0.892	0.05	0.374	0.02	0.607
Leaf width (mm)	0.02	0.592	0.03	0.484	0.02	0.583	0.02	0.739	0.05	0.377	0.01	0.708
Leaf thickness (mm)	**0.82**	**0.000**	**0.20**	**0.050**	0.02	0.542	0.01	0.728	**0.51**	**0.001**	**0.23**	**0.045**
Petiole Length (mm)	**0.44**	**0.003**	0.03	0.522	0.04	0.230	0.09	0.237	**0.41**	**0.004**	0.11	0.178
Leaf Area (cm^2)	0.06	0.330	0.01	0.712	0.03	0.518	0.05	0.379	0.07	0.303	0.04	0.452
Chlorophyll contents (SPAD)	0.09	0.234	**0.37**	**0.008**	0.00	0.868	0.13	0.140	0.04	0.412	**0.28**	**0.025**
Specific leaf area (cm^2/mg)	**0.34**	**0.010**	**0.26**	**0.033**	**0.31**	**0.016**	0.08	0.247	**0.48**	**0.002**	**0.37**	**0.008**

been reported for leaf size in *Populus deltoides* [120] and *Alchemilla alpina* [121], suggesting that diversifying selection with altitude may be responsible for leaf size differentiation. Within each altitude, leaf size was smaller in female than male. Reduced leaf size in females may be due to greater demand for nutrient and carbon for seed and fruit production. Reduced shoot length in females is reported in *H. rhamnoides* [40]. Garden-grown leaves of low altitude origin (2800-3300 m) were smaller in length than those collected from the field in both the gender. However, the opposite trend was observed in plants from higher altitude origin (3500-4200 m). Our result is in agreement with those of Hovenden and Vander Schoor [29] who reported similar trends in *N. cunninghamii*. Increase in leaf size of plants of higher altitude origin (3500-4200 m) in garden experiment may be due to more conducive environmental conditions for plant growth at lower altitude. The results elucidate the phenotypic plasticity in response to change in environmental conditions.

Table 4.4: Two-way ANOVA for leaf morphological characters of *Hippophae rhamnoides* with gender and altitude as main effects

Growing condition	Source	*df*	*F*						
			LL	LW	LT	PL	LA	CC	SLAA
Field-grown	Sex	1	0.775	7.037*	4.413*	1.855	7.456*	1.462	7.274**
	Altitude	3	3.817*	0.354	3.864*	3.298*	2.349	2.971*	12.003***
	SXA	3	0.865	1.670	3.316*	0.347	0.494	0.788	0.292
Garden-grown	Sex	1	0.555	0.315	1.216	0.274	1.094	1.036	0.803
	Altitude	3	0.419	0.949	1.530	0.474	0.714	3.538*	1.788
	SXA	3	0.515	1.421	1.582	0.264	0.737	1.190	1.210

LL= leaf length (mm), LW=leaf width (mm), LT=Leaf thickness (mm), PL =Petiole length (mm), LA= Leaf Area ($cm^{2)}$, CC=Chlorophyll contents (SPAD VALUE), DW=Dry Weight (mg), SLA=Specific leaf area ($cm^{2/}$/mg). Significance: *** = $P < 0.001$; ** = $P < 0.01$; and * = $P < 0.001$

Leaf thickness increased with elevation, which is consistent with trends observed in other species along altitudinal gradients [108-109]. Leaves become thicker with elevation due to increase in intensity of solar radiation and decline in nutrient availability [109]. Increased leaf thickness is an adaptation mechanism against stressful environmental conditions. Leaf thickness is important in terms of carbon assimilation as, so long as light is not limiting, thicker leaves tend to have a higher photosynthetic rate per unit leaf area

[31]. Plants from higher altitudes have higher carbon assimilation rates per unit area [32], and there is a genetic basis for this difference [31], which supports the proposition that thicker leaves would be selected for with increasing altitude. Leaf longevity increased with leaf thickness [110-111] and thus results in an increased residence time of nutrient within the leaves [109], which is beneficial in nutrient-poor environments such as the trans-Himalayan region. Effect of altitudinal gradient on leaf thickness was more prominent in males. Therefore, males are more responsive to change in environmental conditions resulting in greater adaptation. In case of climate change the females are more likely to be adversely affected than males. We found that there is a decrease in SLA with increasing altitude in both the gender in field-grown plants. The result is consistent with most of the earlier studies [26, 29, 112], although a study by Schoettle and Rochelle [113] highlighted an increase in SLA

Table 4.5: Interpopulation variability (coefficient of variation) of leaf morphological traits in field- and garden-grown *H. rhamnoides* in trans-Himalaya

Parameters	Coefficient of variation			
	Male		Female	
	Field-grown	Garden-grown	Field-grown	Garden-grown
Leaf Length (mm)	0.19	0.14	0.17	0.13
Leaf width (mm)	0.21	0.15	0.19	0.13
Leaf thickness (mm)	0.17	0.15	0.21	0.11
Petiole Length (mm)	0.26	0.13	0.21	0.15
Leaf Area (cm^2)	0.32	0.25	0.29	0.19
Chlorophyll contents (SPAD)	0.12	0.12	0.09	0.18
Specific leaf area (cm^2/mg)	0.37	0.33	0.36	0.36

with increasing altitude in *Pinux flexilis*. It has been pointed out that leaves with low SLA generally contain more photosynthetic machinery per unit area [114], increasing water use efficiency and photosynthetic capacity at high altitude [115]. The development of a low SLA is often considered a strategy to increase the longevity of a leaf, in order to optimize the use of scarce nutrients [116-117].Our results showed that most leaf morphological

variation in *H. rhamnoides* is environmentally determined, but SLA and leaf thickness are also dependent on genotype. However, environmental influence was stronger than genetic influence (Table 3). This mix of genetic and environmental influences on morphology of *H. rhamnoides* leaves is also seen in various other species that occur along environmental gradients, including *Metrosideros polymorpha* [33] and *N. cunninghamii* [29].

Petiole length in male increased significantly with increasing altitude ($R^2 = 0.44$), but similar pattern was not observed in female ($R^2 = 0.04$) (Table 3). Elongation of petiole in response to shading is known to increase resource capture under low light condition [118]. However, low light condition alone was an unlikely factor for increased petiole length with increasing altitude in trans-Himalaya. The intensity of solar radiation increases with elevation due to a decline in the optical thickness of the atmosphere [109]. Therefore, the increase in petiole length may be an adaptive response to capture more light to compensate the smaller leaf with increasing altitude. In common-garden experiment, no trend was found for petiole length in both the gender (Table 4.3), suggesting that this trait is essentially an environmental determinism. The chlorophyll contents differed between 'high' and 'low' altitudes, with leaves from higher altitude (3500-4200 m) having more than those from lower altitude (2800-3300 m) in both the gender. Increased chlorophyll contents at higher altitude may be an adaptive mechanism to offset the decline in concentration of CO_2 with elevation. Within each altitude, chlorophyll contents was significantly higher in male than female (Table 4.2), which may be evolutionary advantageous for male for higher photosynthetic activity. Our result is in contrast with that of tropical origin *Piper betle*, where female contained nearly two fold more chlorophyll than male counterparts [119]. Few studies on tree species [122-123] have shown that the field-grown phenotypic variability may result partly from the local genetic adaptations of populations over the altitudinal gradient. In our study, it was observed that variation was always greater in field-grown populations than those in the common-garden experiment, indicating the importance of environmental factors. One of the concerns about the potential confounding of genetic and environmental controls of leaf morphology is that the genetic control may mask the climate signals [124]. This would be evident particularly in the long-lived species. This is unlikely to be the case for *H. rhamnoides* for leaf size characteristics would be useful indicators of environmental conditions.

4.4 Conclusion

It may be concluded from the results of this study that *H. rhamnoides* is a prime candidate to investigate gender response to climate change. The morphological variation in leaves of *H. rhamnoides* is primarily environmentally determined. Our study showed that in the event of climate change the phenotypic plasticity would be a crucial determinant of plant response in mountainous region. Stressful environments will have an added detrimental impact on female than on male. The results elucidated that male will adapt better to the changing climate and may lead to a male-biased population in the event of climate change.

CHAPTER 5
GENDER-SPECIFIC SEASONAL PATTERN AND ALTITUDINAL VARIATION IN FREEZE TOLERANCE RESPONSES IN *HIPPOPHAE RHAMNOIDES*

Abstract

In dioecious plants the adjustment to cold and freeze conditions may differ between male and female individuals. We measured the electrolyte leakage and proline contents in leaves and shoots in male and female *H. rhamnoides* from mid August to mid December. A linear relation between electrolyte leakage and sampling period was observed in both male (R^2=0.871) and female (R^2=0.882) leaves. However, electrolyte leakage in shoot remained constant throughout the sampling period. Proline content in leaves showed a significant increasing trend from August to October followed by a steady decline from November onwards in both the gender. 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. Altitude (3202-3812 m amsl) of plant origin did not have a significant impact on electrolyte leakage in leaves and shoots. Increasing altitude 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). These results 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.

5.1 Introduction

Woody plant species with sessile habitat in regions of temperate environmental conditions are accounted to suffer large seasonal deviations of temperature. Plants adapt to such environmental conditions by growing yearly growth cycle that swaps between shoots that are actively grown and vegetative dormancy by directly synchronizing with the change in seasonal variation [41]. The majority plants are evolved an extent of cold tolerance, the degree of which is usually dependent on blend of the minimum temperature practiced and the duration of experience to cold stress [42]. Overwintering temperate plant species such as SBT are able to tolerate low and freezing temperature. In general, in these species the extent of freezing tolerance is dependent on season and can be modulated by an earlier period of acclimation (pre-hardening) at low but below freezing temperatures, during which large number of changes in morphology, physiology, and at molecular level occur [45]. The full extent of tolerance (hardening) is reached thereafter when plants are exposed to a certain period of low but non-freezing temperature. It has been well documented that accumulation of low-molecular weight compound is observed during exposure to low temperature. Changes in water-soluble sugars [46], in low molecular weight amino acids, especially proline [47], [48], are related with cold acclimation and attainment of frost tolerance. It has been reported that proline might help in regulating the osmotic adjustment and in the protecting of proteins from dehydration [49], stabilization of membrane and also regulate of certain enzyme activities [50] during the course of cold and other stress. A number of evidence appears to specify that membrane structure stabilization and functions play an essential role in determining survival of the species in cold conditions. Certainly, exposure to low temperature can modify the structure and the fluidity membranes owing to alteration by lipid peroxidation [51]. Elevated rates of solute and electrolyte leakage take place in a diversity of tissue injured by chilling stress and they have been used to estimate damage in membrane after chilling stress [125]. Recording the amount of leakage of solutes after cold stress treatments provides an estimate of tissue injury which is a common tool in plant cold hardiness research.

The ability of plant species to adapt and survive in temperatures below freezing point has many facets, which are frequently specific to species, and are due to the result of different cues of environment, despite just low temperature [60]. However, most of the freeze tolerance responses in plants are studied in controlled conditions in growth

chambers. There are significant differences among natural and artificial cold acclimations. Plants which have been cold acclimated in growth chambers may respond differently than those naturally acclimated plants [61], [62]. Changing diurnal temperatures that generate mixed signals in the natural conditions are in distinction to constant rate of temperatures in a growth chamber. Any research intended to investigate and understand cold tolerance should be confirmed in the context of the physiological changes, growth habit and life cycle of the plant species grown under natural field conditions [60].

In dioecious plants 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 reproduction costs involve prioritization of resources available in development of fruit despite in vegetative growth or protection in females as compared to males. A foremost investment in reproductive development is in general related with the difficulties in terms of stress by reactive oxygen and injuries to cells, predominantly under unfavorable conditions [36]. Not accounting for sexual variation could lead to incorrect assessment of a species response to frost. However, to the best of our knowledge studies have not been focused to address the relative importance of the gender of the plant for freeze tolerance in natural conditions particularly in the trans-Himalaya.

SBT is found in an altitudinal range, starting from the sea shores in Europe to more than 4694 m in trans-Himalayan Ladakh. Development of freeze tolerance is strongly influenced by gender and ecotype in this species [126]. 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 cold acclimatize earlier and tolerate to freeze stress higher than females [126], [127]. 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, offers an outstanding platform to examine the relative contributions of environment and gender aspects in relationship between freeze tolerance and altitude. Therefore, the aim of this study was to assess the importance of gender-specific responses of *H. rhamnoides* in cold acclimation and freeze tolerance in trans-Himalayan

environments. Electrolyte leakage and proline contents were taken as 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 are planted in an experimental plot. We expected to find gender differences and strong altitudinal variation for freeze tolerance.

5.2 Materials and Methods

5.2.1 Study site

We collected *H. rhamnoides* subsp. *turkestanica* each 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, elevation 3350 m) on a flat site with direct sunshine at Defence Institute of High Altitude Research (DIHAR) in trans-Himalayan Ladakh, India. The average maximum and minimum temperature during 2014-2015 recorded at our experimental farm at DIHAR was 12.9±8.8°C and -0.2±9.0°C, respectively. The average monthly minimum and maximum are shown in Table 5.1. The mean maximum and minimum relative humidity was 31.0±4.3% and 24.7±3.7%, respectively. The mean annual precipitation was 163 mm.

Table 5.1: Minimum temperature recorded at night at the experimental site in trans-Himalayan region (3350 m amsl)

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

5.2.2 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 sampled on the sun side of the shrub. Fully grown leaves samples and shoot were collected from each branch to measure electrolyte leakage and 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 leaves and shoot samples were collected on 15th of every month from August to December 2015 from each of rooted plants for measurement of electrolyte leakage and proline contents in garden-grown plants.

5.2.3 Electrolyte leakage

The shoot electrolyte leakage test was carried out using a method described earlier by Houimli et al. [128] with slight modification. The leaves and shoots (0.1 g) were washed twice with tap water and subsequently with deionized water after that immersed in test tube with 25 ml deionized water. The tubes were capped, agitated and then allowed to incubate at room temperature 25°C. The conductivity (EC1) of the solution was measured after 24 h using conductivity meter (SensION^{+} EC71, HACH, Barcelona). The leaf and shoot samples were then autoclaved for 1 h to release all electrolytes. The solution was allowed to cool to room temperature before taking second conductivity (EC2) reading. The electrolyte leakage (EL) was measured using the formula EL= EC1/EC2×100.

5.2.4 Proline contents

The proline content was determined using the method described earlier [129]. Proline was extracted from 0.5 g fresh weight plant sample, grinded properly in 10 ml of 3% aqueous sulfosalicylic acid and the mixture was separated through Whatman # 2 filter paper. To the 2 ml of filtrate 2 ml acid-ninhdrin and 2 ml of glacial acetic acid were added in a test tube and incubated for 1 h at 100°C, and the reaction was stopped using ice bath. The reaction mixture was extracted using 4 ml toluene, vigorously mixed for 15-20 sec. The chromospheres with toluene were separated from the aqueous phase, brought to room temperature and the absorbance at 520 nm was recorded in a 96 wells micro-plate reader (SpectroMax M2 e, Molecular Devices, Sunnyvale, CA, United States) taking toluene for the blank. The concentration of proline was determined on the basis of calibration curve and calculated on the basis of fresh weight as follows:

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

5.2.5 Statistical analysis

In all the experiments the samples are the mean of three replicates and values were considered to be significant if P-values were ≤ 0.05. All statistical analysis was performed using SPSS (Statistical Program for Social Sciences, SPSS Corporation, Chicago, Illinois, USA). The analysis were done through one-way ANOVA and the differences of the mean were compared using post hoc analysis with 2-sided Tukey's HSD tests to ensure significant differences between different months. Student's t test was performed to compare significant difference between male and female in each month.

5.3 Results and Discussion

5.3.1 Gender-specific seasonal pattern in freeze tolerance

Seasonal pattern in acclimation and freeze tolerance is presented in Table 5.2. Electrolyte leakage and proline contents in leaves and shoots varied significantly during the sampling period. Significant increase in electrolyte leakage was observed in leaves during mid October when the incidents of temperature below 5°C was observed on 25 days between mid September and mid October (Table 5.2). The linear regression showed almost linear relationship between electrolyte leakage and sampling period in both male (R^2=0.871) and female (R^2=0.882) leaves. However, electrolyte leakage in shoot remained constant throughout the sampling period and no linear relationship was observed in both the gender. Within each sampling period, there was no significant role of gender on electrolyte leakage. The shoot electrolyte leakage was significantly higher than that of leaves in August and September. However, the opposite trend was observed in November and December in both the gender.

The proline contents in leaves showed a significant increasing trend from August to October followed by a steady decline from November onwards (Table 5.2) in both the gender. However, the shoot proline contents showed a steady increasing trend from August to December. 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< 0.001, Student's t-test). However, in shoot the proline

contents remained significantly higher in male than female from October to December (Table 5.2). Throughout the sampling period the proline contents in shoot remained significantly higher than that in leaves except in October.

Table 5.2: Seasonal pattern in electrolyte leakage and proline contents as a measure of cold hardiness in *H. rhamnoides*

Tissue	Date	Electrolyte Leakage (%)		Proline Contents (μM g^{-1}FW)	
		Male	Female	Male	Female
Leaves	15 Aug	12.8±4.3^{a}	14.5±4.5^{a}	0.3±0.2 ^{a}	0.3±0.2^{a}
	15 Sept	12.4±10.9^{a}	11.6±4.2^{a}	26.8±17.1^{b}	32.7±23.5^{ab}
	15 Oct	38.5±11.8^{bc}	39.0±11.3^{b}	234.3±76.0^{d}***	130.7±38.9^{cd}
	15 Nov	65.1±13.6 ^{c}	62.8±6.6^{c}	71.3±23.5^{c}	69.1±20.9^{b}
	15 Dec	61.5±13.2^{c}	62.9±13.2^{c}	46.9±13.7^{bc}	31.8±7.1^{ab}
Shoot	15 Aug	35.4±17.6^{bc}	33.8±20.3^{bc}	0.6±0.4^{a}	0.5±0.4^{a}
	15 Sept	31.8±4.6^{b}	30.4±10.0^{b}	88.1±72.0^{c}	91.0±62.7^{bc}
	15 Oct	32.3±12.6^{bc}	36.5±14.8^{bc}	219.2±135.9^{d}***	108.5±77.7^{bc}
	15 Nov	35.2±8.6^{b}	33.2±16.9^{ab}	237.5±113.1^{d}*	164.0±120.4^{c}
	15 Dec	37.4±11.1^{bc}	37.1±9.5^{b}	361.7±158.6^{d}*	298.2±132.5^{d}

Values represented as mean ± SD. For each column, different lowercase letters indicate significantly different at $p < 0.05$, 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 $p \leq 0.001$

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. Different sessile woody perennial plants acclimate differentially to a specified range of temperature [130]. In *Weigela* some cultivars acclimate late, with considerable hardening that take place along with the minimum air temperature decreasing below 5°C on quite a few occasions [131]. Likewise, in two populations of *Leptospermum scoparium* the perceptible threshold temperature for the commencement of freeze hardening was about 6°C [132]. Exposure of *Rhododendron* to 5°C in controlled condition is reported to confer cold tolerance [133]. Significant degree of increase in electrolyte leakage was recorded in leaves during mid October when temperature below 5°C was

observed on 25 days between mid September and mid October. Cold hardening occurred during the period as marked by significant increased 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.

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 male indicates that males contain a better osmoregulation adjustment as compared to females, since proline is an important osmoregulation compound in the leaves of many plants [134]. Proline is essential in protecting protein from denaturation, an active source for carbon and nitrogen, and for acting as a free radical scavenger [135], [136]. The increased proline found in male indicates better protection to stress from environmental source in male cells than those to female cells. These results suggest that response to freezing by gender is significant and that male sexes exhibit a better self defense mechanism as compared to females in *H. rhamnoides*. The greater cold tolerance of leaves of male plants than females may be beneficial in terms of cold tolerance of the whole plant. Lennartsson and Ögren [137] suggested that deciduous trees even may get advantage from upholding leaves as long as feasible throughout autumn to allocate photosynthesis in continuous, which may be significant in structuring reserves required 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.

Plant organs vary in their extent of tolerance; usually the leaves are much more sensitive as compared to shoots. Leaves develop freezing tolerance faster than shoots when they are exposed to low temperature. Our data clearly imply that leaves of SBT are more susceptible to the environmental cues triggering acclimation as compared to the shoot tissues. The result is in consistent with findings of Li et al. [138] where buds and leaves of Silver birch (*Betula pendula*) are found to show higher response to the cues of environment as compared to stem tissue.

5.3.2 Altitudinal variation in freeze tolerance

Altitude of plant origin did not have a significant impact on leaves and shoot electrolyte leakage in both male and female *H. rhamnoides* (Table 5.3, 5.4). Similarly, no increasing or decreasing trend was observed in leaf proline contents with increasing altitude (Table 5.3). 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< 0.01, Student's t-test). The results suggested that plants from higher altitude possess more effective mechanisms that prevent the frost damage. Linear relationship between increased proline contents and increasing altitude supported the findings. This result is in consistent with findings of Greer and Robinson et al. [132] 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* [139].The overall proline contents in both leaves and shoot was significantly higher in male (112±77, 143±66 µM g^{-1}, respectively) than female (87±46, 119±82 µM g^{-1}, respectively). Within each sampling site, electrolyte leakage was significantly higher in leaves than shoot irrespective of the gender. Opposite trend was observed in proline contents in both the gender.

Table 5.3: Altitudinal variation in electrolyte leakage and proline contents in shoot as a measure of cold hardiness in *H. rhamnoides* in December under natural field condition

Altitude (m asl)	Electrolyte Leakage (%)		Proline Contents (µM g^{-1} FW)	
	Male	Female	Male	Female
3203±5.6	31.2±2.9^{a}	31.3±3.7^{a}	84.2±43.4^{a}	59.6±56.6^{a}
3239±5.0	38.8±6.2^{a}	39.5±6.1^{b}	113.6±48.2^{ab}	73.0±43.6^{a}
3260±4.6	33.0±16.6^{a}	29.2±5.1^{a}	121.3±47.4^{ab}	96.6±66.1^{abc}
3340±8.7	32.8±14.0^{a}	33.2±9.0^{ab}	175.1±75.8^{b***}	75.0±19.3^{ab}
3464±23.9	30.1±5.5^{a}	26.6±3.7^{a}	180.5±65.9^{b}	163.8±53.8^{bcd}
3636±49.6	31.1±4.7^{a}	32.7±5.2^{ab}	165.0±64.4^{b}	166.3±98.8^{cd}
3812±24.8	31.6±7.0^{a}	32.1±4.7^{ab}	159.6±59.0^{ab}	195.7±90.8^{d}
Mean	32.7±9.4	32.1±6.6	142.9±65.7***	118.6±81.5

Values represented as mean ± SD. For each column, different lowercase letters indicate significantly different at $p < 0.05$, as measuredby 2-sided Tukey's HSD

***Value significantly higher than that of opposite sex at $p \leq 0.001$.

5.4 Conclusion

Our results exhibited different responses of male and female of *H. rhamnoides* to environmental cues in natural conditions. Female plants suffer more from harmful effects of freezing as compared to males. Leaves develop freezing tolerance more quickly than shoots when exposed to cold temperature. It is, therefore, suggested that when *H. rhamnoides* is planted in colder region, females be provided with an added protective measures to improve their chances for survival.

CHAPTER 6
SSR MARKERS BASED GENETIC DIVERSITY CHARACTERIZATION AND MARKER-TRAIT CORRELATION FOR ANTIOXIDANT PROPERTIES IN MALE AND FEMALE POPULATIONS OF *HIPPOPHAE RHAMNOIDES*

Abstract

SBT is a dioecious plant wherein female plant produces fruits. Currently, most of commercial demands of SBT are fulfilled by extraction of the wild population causing reduction of its natural resources. Despite the recent studies using morphological and physiological traits, the genetic diversity targeting male and female populations at molecular level have not been studied. In this study, 32 polymorphic SSR markers based evaluation of male and female populations (N: 180, six population) revealed high level of overall genetic diversity (GD) with higher GD in of male populations (I=0.770, He=0.471) as compared to female populations (I=0.753, He= 0.460), although not significant. The result suggested current ecological environment started causing selection pressure to females and the plant need attention for effective conservation of genetic resources. High gene flow (*Nm*: 3.4), low genetic differentiation (F_{ST}: 0.068) and high within population genetic variation (93%) indicates that most of the genetic diversity is limited within population. Furthermore, neighbor joining and Bayesian-based STRUCTURE analysis exhibited an intermixing in the clustering of male and female populations whichmight indicate the variation observed between them are controlled by autosomal genes in response to different environment and not sex-linked genes. Correlation of molecular data using Multiple Regression Analysis (MRA) revealed that 29 alleles corresponding to 8 SSR markers were found to be correlated with phytochemical attributes. Among these, 5 and 11 SSR loci were positively correlated with different phytochemical attributes (TPC, FRAP and DPPH) of male and female populations, respectively. Thus, inferences of the current study can be used for selection of male plant to strategize the commercial cultivation, genetic improvement and implementation of conservation plan for SBT.

6.1 Introduction

The discrete individuals of a particular species are not genetically alike. Their DNA sequences diverge to an extent and these differences leads to the source of genetic diversity, well-known as polymorphism, of a species [140]. Understanding genetic variations are pre-requisite for implementation of conservation and management programme for preserving valuable populations [141], [142], [143], [144], [145]. Genetic diversity in plant species is the basis for their survival and adaptation to counter evolutionary forces and to some extent, human survival is also dependent on it [146]. Dioecy in plants are an imperative part of both terrestrial and forest ecosystem [67] which contributes to about 5-6% of plant species represented by 157 flowering plant families and 959 genera [147]. Genetic diversity of dioecious plant is greatly influenced by mating pattern, floral morphology and method of reproduction [148]. It can provide information about genotypic distribution of populations in natural along with their reproductive success [149], [150], [151]. Furthermore, insight knowledge about the genetic diversity of each gender will enhance the chances to obtain a new genetic combinations [72], which will enable us to breed of new varieties [67]. In general, the outcrossing mating system assists plant populations to preserve high levels of genetic diversity in comparison to self-pollinated plant species, wherein self-pollinated species is supposed to maintain half of the total genetic diversity [152]. Moreover, inbreeding and outbreeding plant species not only differ in overall genetic diversity levels [153], but they may also differ in the scale of within population and between population variances of genetic diversity [154]. Species tolerance and adaptations may affect population genetic diversity, particularly the genetic diversity of sexual populations [155], [156], [157]. Differences in the two gender appears due to resource allocation biases between them [40], [36] wherein, males invest more energy in chemical or/and structural defenses while females invest more energy in reproduction [158]. Females are often less adaptive as compared to males in stressful environment [159], [160], [161] while there are some exceptions where some females can also be more tolerant to adversity than males [162], [163]. However, studies on sexual differences generally focused on the physiological adaptation differences in males and females, merely a little studies explore the genetic diversity of male and female populations separately at molecular genetics level [71], [164], [67].

Till date, the genetic diversity studies in *H. rhamnoides* have been carried out using combined not defining the male and female populations, separately [69], [165], [68]. Gender specific GD inferences using male and female populations with considerable high number of individuals (N: 180) has not been reported. Despite its high medicinal importance, gender specific genetic diversity has not been studies at molecular level. Largely, efforts have not been made to establish marker-trait correlations, which could be otherwise having great implications for selection of superior genotypes from the natural resources. Therefore, in the present study, an effort has been made to characterize the natural diversity of male and female populations at molecular level. A total of 180 genotypes (90 each of male and female genotypes) representing six natural populations of trans-Himalayan Ladakh were evaluated using SSR markers and antioxidant traits. Furthermore, efforts were also made to establish correlation of SSR markers and different antioxidant traits [total phenolic content (TPC), ferric reducing antioxidant potential (FRAP), and 2, 2-diphenyl-1-picrylhydrazyl (DPPH)].

6.2 Materials and Methods

6.2.1 Sample collection and DNA extraction

Fresh leaf sample of 180 individuals (15 male and 15 female each) were collected from six different collecting sites of trans-Himalayan Ladakh and dried in silica gel for DNA isolation. Genomic DNA was isolated using CTAB method by Doyle and Doyle [166] with slight modifications. The concentration DNA was measured with the help of Nanodrop (Thermo Scientific) and purity was checked on 0.8 % agarose gel. The DNA sample was diluted to the final concentration of 25-30ng /µl before PCR amplification. Screening and genotyping of 96 designed genic microsatellite markers was performed at standard PCR reaction with suitable annealing temperatures of primer pairs. Amplified DNA fragments were separated on 6 % denaturing urea-PAGE and visualized with the help of silver stain as performed by Bhandawat et al. [167].

6.2.2 SSR analysis

A total of 96 SSR primers derived from in-house transcriptome data available with collaborator (CSIR-IHBT) were utilized for genetic diversity analysis. The validation of 96 primers with 12 individuals (2 from each population) revealed that 82 SSR were polymorphic. Further 32 polymorphic SSRs were used for the diversity characterization of

180 individuals belonging to six populations. Further, the genotypic data was also used for association with previously performed antioxidant analysis data of these 180 individuals (First 15 male and female individuals of each population) (Objective 2).

6.2.3 Data analysis

The reproducible SSR alleles were scored on the basis of co-dominant scoring method in order to capture heterozygosity nature of the marker. GenAlex*version* 6.5 software [168] was applied to observe genetic parameters: percentage of polymorphic loci (%P,) Number of observed alleles (Na), Number of effective alleles (Ne)**,** Shannon's information index (I), Observed heterozygosity (Ho)**,** Expected heterozygosity (He)**,** Fixation index (F), analysis of molecular variance (AMOVA), genetic differentiation index F_{ST} and gene flow (Nm). Darwin 5 ver. 5.0.158 was employed to create a Neighbour Joining (NJ) tree on the basis of Jaccard coefficient utilizing Unweighed Pair Group Method with Arithmetic Mean (UPGMA) [168]. Further, Bayesian based clustering was carried out with the help of software: STRUCTURE v 2.3.4 in order to assess the genetic structure of all populations [169], [170]. This clustering method uses the Markov Chain Monte Carlo (MCMC) algorithm. A total of 10 independent runs for each K value ranging from 2 to 8 along with burn-in period of 100,000 and the Markov Chain Monte Carlo (MCMC) repeats after burn-in at 100,000 were executed. Statistical significant test were performed with the help of paired-sample *t*-test using Microsoft excel.

6.2.4 Marker trait correlation analysis

To discover informative markers linked with the different antioxidant activity in SBT, Multiple Regression Analysis (MRA) was performed to correlate molecular data and biochemical data as independent and dependent variables respectively. Beta value delineate the standardized regression coefficient (BS_x/S_y), where B indicates regression coefficient (slope) while Sx and Sy are the standard deviations of independent (x) and dependent (y) variables [171], [172]. Associated of the trait in consideration with SSR loci were considered to be significant at p values 0.5 or 0.1 or 0.001 levels.

6.3 Result and discussion

6.3.1 Overall, and male and female genetic diversity

Genetic diversity study plays very important role in survival and adaptation of a species. It makes possible for species to adapt any change environment, and high genetic

diversity helps to maintain stable evolution and widen the distribution range for a species [173], [144] especially in long lived species. For diversity, evolutionary and phylogenetic studies, simple sequence repeat markers (SSRs) are preferred over dominant markers (RAPD, AFLP, and ISSRs) because SSRs have multiple advantages, such as multi allelic, highly polymorphic, genome wide distribution and co-dominant natures [174], [167], [175]. Implementation of SSR makers for gender specific genetic diversity characterization is limited in SBT. SSR marker used in our study provided insight on genetic diversity, population structure and marker trait correlation. 94 alleles obtained from 32 SSR markers were sufficient to genetically distinguishing phenotypically similar 180 SBT individuals as reported in previous reports [176], [177], [178].

Table 6.1 explicit the values of different GD estimators in male and female of SBT. The 32 polymorphic SSR markers exhibited 94 alleles in 180 individuals of SBT with a mean value of 2.641observed alleles at each locus. The mean effective allele (Ne) was recorded to be 2.061 per locus. Shannon's information index (I) varied from 0.708 to 0.922 with a mean I of 0.762, while average observed heterozygosity (Ho) and expected heterozygosity (He) were 0.279 and 0.466, respectively. Our data revealed that overall genetic diversity of populations was high (I=0.766, He=0.522) as reported previously in dominant AFLP markers [68] which indicates high adaptability of the species. Dioecious plant species have primarily evolved to avoid inbreeding [179] and outcrossing species express higher genetic diversity. High genetic diversity reported in the male and female populations suggests that selection of potential populations for implementations of genetic improvement programme as observed in dioecious *Populus tremuloides* [180], *Myrica rubra*[71], [181], [182]. In addition, lower *Ho* than *He* suggested a deficiency of heterozygotes in these populations which might be explained due to limited dispersal of pollen approx 15-20m [183], [184] thus in *ex-situ* conservation more cross pollination should be promoted. Although not statistically significant, the average diversity was relatively higher in male (I=0.770, He=0.471) as compared to female (I=0.753, He=0.460). High diversity estimates revealed by SSR markers in this study were found to be higher than previously studies in SBT [165] possibly due to bigger sample size in this study. The current observation was consistent with earlier studies in *Myrica rubra* [71] that no significant difference was observed between genetic diversity of males and females. The males and female populations diversity recorded a significant distinctness at morphological and physiological level [40].

Table 6.1: Genetic diversity of male and female subpopulations in *H. rhamnoides*

	Pop	%P	Na	Ne	I	Ho	He	F
Male	Spituk	100	2.714	2.182	0.820	0.286	0.504	0.399
	Shey	100	3.000	2.223	0.876	0.219	0.517	0.530
	Chuchot	100	2.714	2.101	0.774	0.267	0.467	0.448
	Phyang	100	2.143	1.767	0.579	0.305	0.375	0.256
	Horzey	100	2.857	2.103	0.824	0.124	0.491	0.758
	Sakti	85.71	2.571	2.085	0.750	0.333	0.474	0.287
	Average	**97.62**	**2.667**	**2.077**	**0.770**	**0.256**	**0.471**	**0.446**
Female	Spituk	100	3.143	2.126	0.844	0.238	0.489	0.531
	Shey	100	2.857	2.149	0.832	0.286	0.507	0.472
	Chuchot	85.71	3.143	2.355	0.922	0.286	0.552	0.496
	Phyang	85.71	2.429	1.993	0.715	0.276	0.454	0.488
	Horzey	85.71	2.571	1.984	0.708	0.333	0.444	0.278
	Sakti	71.43	2.143	1.660	0.496	0.390	0.316	-0.057
	Average	**88.10**	**2.614**	**2.045**	**0.753**	**0.302**	**0.460**	**0.368**

%P=percentage of polymorphic loci, Na, Number of observed alleles; Ne**,** Number of effective alleles; I, Shannon's information index; Ho**,** Observed heterozygosity; He**,** Expected heterozygosity; F= Fixation index.

Besides, the SSR being more stable marker as compared to dominant marker, diversity estimators also revealed higher in male as compared to female populations although statistically not significant. It appears that current ecological environment started causing selection pressure to females and the plant need attention for effective conservation of genetic resources. Additionally, the genetic parameters of populations from low altitudes (I=0.845, He=0.506) were relatively higher than that from high altitudes (I=0.679, He=0.426). High level of average GD in Indus belt (low altitude) than populations separated by geographical barriers (high altitudes) suggests better opportunity for selection of the population for commercial cultivations from the belt. Further, along the process of *ex situ* conservation, artificial crossing or/and planting of distantly related plants should be carried out in order to improve the diversity of high altitude populations.

6.3.2 Genetic differentiation, gene flow and structure of male and female populations

Overall genetic differentiation was low with an average F_{ST} value of 0.068 and high gene flow (*N*m 3.4). Further, Analysis of Molecular Variance (AMOVA) also showed that the major part of total variance (93%) resides within population (within individual=53%, among individual= 40%) and only 7% of variance were attributable among populations (Table 6.2 and Figure 6.1). Thus, AMOVA study suggests that *Hippophae* SSR marker has explained a better partitioning of variation and the population of SBT are panmictic without any genetic divergence.

Table 6.2: Total genetic variance analysis (AMOVA) of *H. rhamnoides* brought from SSR results.

Source	Df	SS	MS	Est. Var.	%
Among Pops	11	68.331	6.212	0.125	7%
Among Indiv	168	411.467	2.449	0.737	40%
Within Indiv	180	175.500	0.975	0.975	53%
Total	359	655.297		1.838	100%

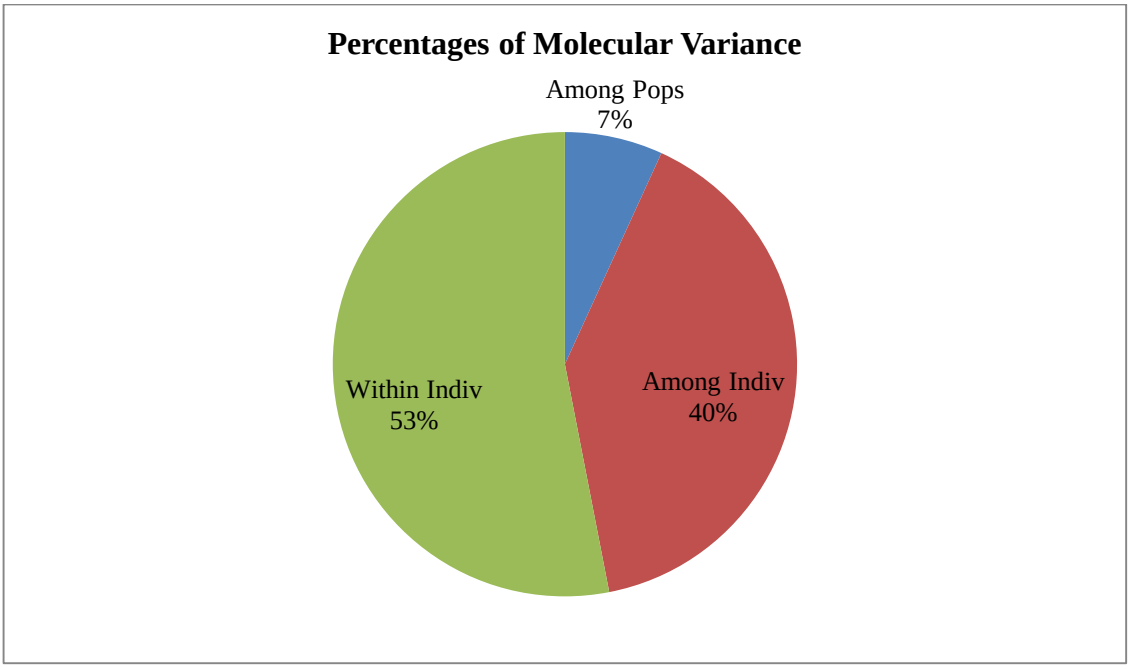


Figure 6.1: Total genetic variance analysis (AMOVA) of *H. rhamnoides* brought from SSR results.

Low differentiation may possibly be related to the mating system and life cycle of *H. rhamnoides*. Generally, in long-lived woody, wind-pollinated, out-crossed and dioecious species the differentiation among populations is significantly low [140], [185], [186]. Additionally, pairwise *FST* values (Table: 6.3) indicated no statistically significant differentiation between male and female population which could be due to the reason that sexual differentiation took place earlier than genetic differentiation [71]. UPGMA based dendrogram (Figure 6.2) clustered all the 180 individuals in three separate clusters with no clear differentiation between populations of different geographical origin. Furthermore, clustering carried out with Bayesian model based STRUCTURE analysis (Figure 6.3.b) revealed a similar pattern and all the six populations could be classified into three clusters (K = 3), best fit the dataset (Figure 6.3.a), indicating that three distinct genetic pools existed in *H. rhamnoides* populations of Ladakh region. None of the populations constituted a distinct group but remained intermixed in three clusters.

Table 6.3: Pairwise F_{ST} values among ten male and female subpopulations

	SptM	SptF	ShyM	ShyF	ChuM	ChuF	PhyM	PhyF	HorM	HorF	SktM	SktF
0.00												SptM
0.03	0.00											SptF
0.02	0.04	0.00										ShyM
0.02	0.02	0.03	0.00									ShyF
0.04	0.05	0.06	0.03	0.00								ChuM
0.02	0.04	0.03	0.01	0.04	0.00							ChuF
0.09	0.09	0.09	0.09	0.10	0.09	0.00						PhyM
0.06	0.05	0.06	0.05	0.06	0.05	0.05	0.00					PhyF
0.06	0.05	0.07	0.06	0.07	0.05	0.04	0.03	0.00				HorM
0.07	0.07	0.09	0.07	0.07	0.07	0.03	0.02	0.03	0.00			HorF
0.05	0.05	0.07	0.06	0.06	0.07	0.09	0.04	0.04	0.05	0.00		SktM
0.10	0.14	0.13	0.11	0.13	0.11	0.07	0.08	0.10	0.08	0.09	0.00	SktF

SptM (Spituk male), SptF (Spituk female), ShyM (Shy male), ShyF (Shy Female), ChuM (Chuchot male), ChuF (Chuchot female), PhyM (Phyang male), PhyF (Phyang female), HorM (Horzey male), HorF (Horzey F), SktM (Sakti male), SktF (Sakti female).

The mixing of populations could be due to many reasons; seeds of SBT can easily be dispersed to far-off regions by birds or anthropogenic intervention that leads to intermixing of populations. Furthermore, SBT is grazed by domestic animals; thereby, causing distinct flow of pollen/seed to regions, thus causing mixing of populations. Besides, the local dwellers use SBT as biofencing due to its thorny nature thus anthropogenic causes further aid in intermixing of the populations. Additionally, significant amount of gene flow occurred in the region sampled from natural populations, therefore, suggesting no population groups separately.

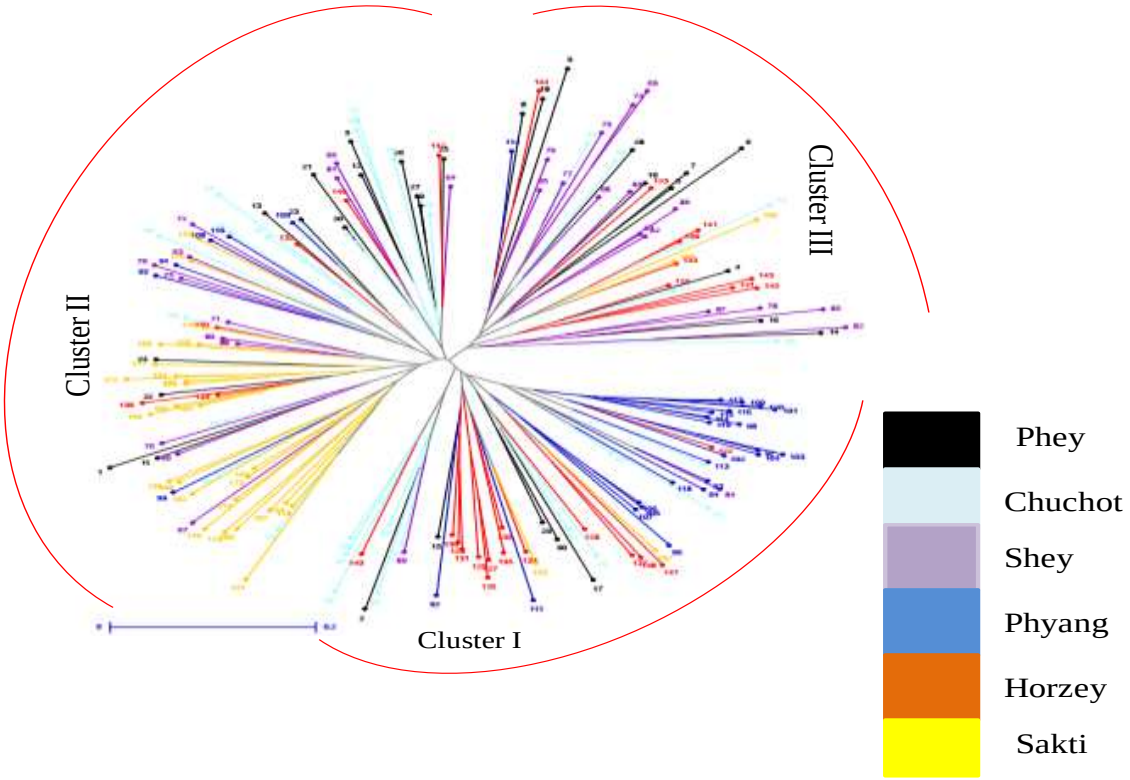
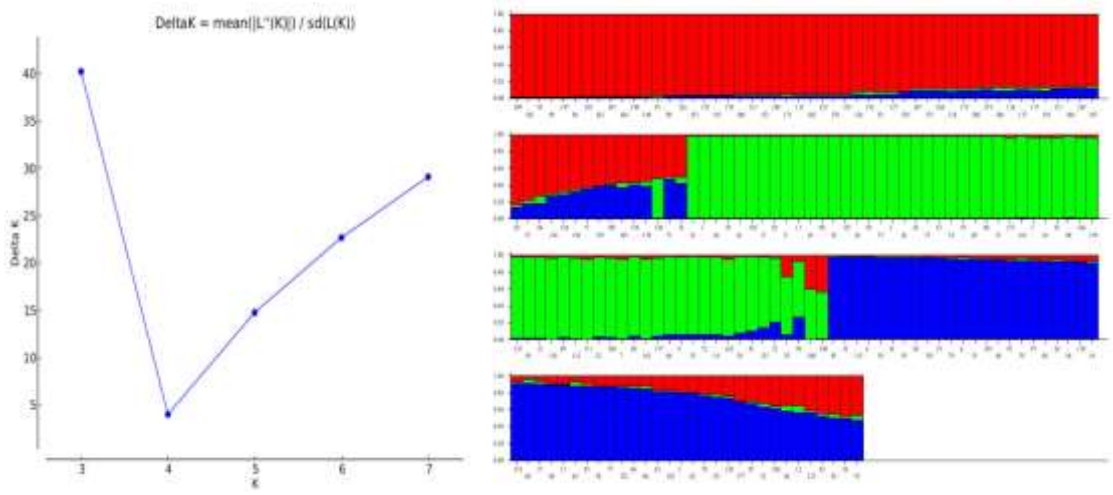


Figure 6.2: Bootstrapped neighbor–joining (NJ) tree of 180 *H. rhamnoides* accessions using 32 SSR markers

However, Ladakh being a major distribution site of *Hippophae* (70% of the total SBT in India) and overexploitation of the crop for berries and leaves in the region demands conservation and proper management of *Hippophae*. Thus, during *ex situ* conservation, appropriate sampling strategies of genetic resources should be undertaken. Moreover, STRUCTURE result had little relationship with sex, which is consistent with previous research in *S. viminalis* [67]. This suggests male-female populations co-evolved from hermaphrodite [187] and the variation observed between them are controlled by autosomal genes in response to different environment and not sex-linked genes. Current inferences on



(a) (b)

Figure 6.3: STRUCTURE analysis based clustering of 180 individuals of *H. rhamnoides.* (a) Plot showing population size and corresponding value of delta K, (b) histogram showing two genetic pools in population-wise clustering.

genetic diversity and population structure provide basic genetic profile for developing appropriate sampling strategies for optimization and implementation of *ex situ* conservation in *H. rhamnoides* genetic resources for appropriate management of SBT natural populations.

6.3.3 Marker trait correlation in male and female subpopulations

Stepwise Multiple Regression Analysis (MRA) was used to establish correlation between SSR markers and antioxidant activity (TPC and FRAP assay) among 180 individuals. MRA provides the coefficient of determination which indicates the proportion of variability of a dependent variable that can be explained by a linear function of independent variables [188]. This approach has been utilized to correlate molecular marker with morphological and biological traits in many plant species. Overall summary of MRA and different coefficients for antioxidant are presented in Table 6.3. The MRA identified 29 SSR loci corresponding to 8 SSR markers, significantly [29] correlating (positively/ negatively) with antioxidant activities. Broadly, one SSR locus (HR_T_9E) in male and three SSR locus (HR_T_8B, HR_T_9D, HR_T_9E) in female exhibited a positive correlation with TPC while, two SSR locus (HR_T_4B, HR_T_6B) in male and four SSR locus (HR_T_2D, HR_T_5A, HR_T_5B, HR_T_8D) in female exhibited a negative correlation with TPC.

Table 6.4: SSR markers or alleles associated with antioxidants in *H. rhamnoides* as revealed by MRA

Antioxidant Activity	SSR markers	Male			Female		
		β-coefficient	t value	p value	β-coefficient	t value	p value
Total phenolics content	HR_T_2D	-	-	-	-0.278	-2.711	0.008
	HR_T_4B	-0.220	-2.188	0.037	-	-	-
	HR_T_5A	-	-	-	-0.255	-2.479	0.015
	HR_T_5B	-	-	-	-0.337	-3.357	0.001
	HR_T_6B	-0.118	-1.779	0.015	-	-	-
	HR_T_8B	-	-	-	0.212	2.033	0.045
	HR_T_8D	-	-	-	-0.285	-2.789	0.006
	HR_T_9D	-	-	-	0.223	2.148	0.034
	HR_T_9E	0.211	2.112	0.046	0.235	2.267	0.026
FRAP reducing antioxidant assay	HR_T_1A	-0.453	-2.355	0.021	-	-	-
	HR_T_1B	-	-	-	-0.252	-2.244	0.017
	HR_T_2B	0.308	3.042	0.003	0.28	2.736	0.008
	HR_T_2D	-0.210	-2.01	0.047	-0.325	-3.266	0.002
	HR_T_3C	-	-	-	-0.218	-2.1	0.039
	HR_T_3D	-	-	-	0.255	2.162	0.033
	HR_T_4B	-0.25	-2.427	0.017	-	-	-
	HR_T_5D	-0.34	-3.369	0.001	-0.415	-4.275	0.000
	HR_T_6A	-0.212	-2.035	0.045	-	-	-
	HR_T_9C	-0.327	-3.243	0.002	-	-	-
	HR_T_9D	-	-	-	0.259	2.513	0.014
	HR_T_9E	0.290	2.845	0.006	-	-	-
DPPH assay	HR_T_1B	-	-	-	0.229	2.21	0.03
	HR_T_2C	-	-	-	-0.251	-2.433	0.017
	HR_T_2D	-	-	-	0.385	3.912	0.000
	HR_T_3C	-	-	-	0.218	2.091	0.039
	HR_T_4B	0.257	2.495	0.014	0.255	2.168	0.033
	HR_T_5D	-	-	-	0.336	3.343	0.001
	HR_T_6A	0.32	3.168	0.002	-	-	-
	HR_T_9D	-	-	-	-0.026	-1.977	0.051

Similarly, two SSR locus (HR_T_2B, HR_T_9E) in male, and three SSR locus (HR_T_2B, HR_T_3D, HR_T_9D) in female explicated a positive correlation with FRAP while, six SSR loci (HR_T_1A, HR_T_2D, HR_T_4B, HR_T_5D, HR_T_6A, HR_T_9C) in male and four SSR loci (HR_T_1B, HR_T_2D, HR_T_3C, HR_T_5D) in female explicated a negative correlation with FRAP. In terms of DPPH assay two SSR loci (HR_T_4B, HR_T_6A) in male and five in female (HR_T_1B, HR_T_2D, HR_T_3C, HR_T_4B, HR_T_5D) indicated positively correlation while two SSR loci (HR_T_2C, HR_T_9D) in female indicated negative correlation with the antioxidant assay. MRA studies are efficiently utilized in other plants, for instance, Jugran et al. [189] correlated three ISSR markers with DPPH assay in *Valeriana jatamansi*, whereas, Khub et al. [73] reported thirty eight SSR alleles and 135 RAPD fragments were found associated with 14 traits affecting fruit quality. With respect to SBT, studies have been conducted to correlate dried shrink disease [79] and fruit oil content [80] of *Hippophae* with ISSR markers. However, correlation of antioxidant property of male and leaves with molecular marker is a first approach for this crop. Interestingly, 5 and 11 SSR loci were found to be positively correlated with different phytochemical attributes of male and female populations, respectively. Nevertheless the compositions of total amount antioxidant compounds are controlled by multiple genes. However, for plants with no prior information, traits specific correlated markers identified in present study can be validated in multiple environmental conditions and could be used as putative markers for selection of promising individuals/ populations to met commercial demands.

6.4 Conclusion

Current inferences on genetic diversity and population structure will allow in development of appropriate sampling strategies for optimization and implementation for conservation in *H. rhamnoides* genetic resources. Information generated from highly informative SSR marker in *H. rhamnoides* natural population revealed that the current study possibly be utilized to strategize the commercial cultivation, genetic improvement and implementation of selection of elite individual/ genotype in its major distribution site in India.

SUMMARY

Development of an improved vegetative propagation method and gender difference in rooting success

Recent interest in the vegetative propagation of SBT arises because of increasing demand for SBT. Vegetative propagation of SBT through pencil thickness (5-10 mm diameter) cuttings is the method of choice for propagation. However, a more efficient mass propagation system is required for successful plantation of SBT. In this study we made effort to develop an improved vegetative propagation method and study the effect of the plastic mulching, coloured shade netting, spacing, cutting thickness and gender differences on rooting and growth of SBT. One year old growth stem cutting (2.9±0.8 mm thickness) resulted in 97.6±2.2% rooting success in greenhouse condition. Use of rooting hormone did not have any significant result. We observed that use of silver black plastic mulching film resulted in 10% higher rooting success and significant plant growth of pencil thickness cuttings in open field condition. Suppression of weed emergence by the plastic mulch resulted in 75.8% time saving in manual weeding by farm workers. Reduction in light intensity by 66% using green shade net resulted in significant reduction in rooting and growth of nursery plants. Three different spacing between cuttings did not show significant difference in rooting and growth related parameters suggesting that cuttings can be planted denser (3"×3") under mulching to get higher number of nursery stock per unit area. Cutting thickness showed significant effect on rooting success. Highest rooting percentage was observed in pencil thickness cuttings (7.5±1.6 mm diameter) followed by cuttings with 2.9±0.8 mm and 11.3±1.7 mm basal diameter in open field condition. Gender did not showed significant difference in percent rootability. However, shooting parameters like shoot length, number of primary root and basal diameter of secondary root was found to be higher in male than that of female. Present study could assist in generation of a vegetative propagation technique wherein faster growth, larger number of cuttings can be propagated with higher rooting success rate.

Gender differences and seasonal variation in total phenolics and antioxidant capacities in Seabuckthorn leaves

SBT leaves are reservoir of important health promoting bioactive compounds and are used for product development. In this study we made an attempt to delineate the gender differences and harvesting season on total phenolic content (TPC) and total antioxidant capacity (TAC) in SBT leaves. We collected leaf samples that comprised of 200 plants

(100 ♂ and 100 ♀) from six natural populations and carried methanolic and acetone extraction for quantification of TPC and TAC. Significantly lower TPC (95.0±23.8 mg GAE/g DW) was observed in females as compared to males (100.8±23.9 mg GAE/g DW). Likewise, significantly lower antioxidant activity in terms of FRAP was detected in females (6.1±1.2 Fe^{2+} mmol/g DW) as compared to males (6.5±1.1 Fe^{2+} mmol/g DW). Significant increase in TPC was observed in male leaves from July to October followed by a significant decrease in November. However, increase in TPC was observed up to August in female leaves and then showed steady declining trend. Similar trend was observed in TAC in both the gender except that female also showed increasing TAC from July to October. October is the best time to harvest SBT leaves, and that leaves contain significantly higher hydrophilic than lipophilic phenolics and antioxidants.

Gender differences in phenotypic plasticity and adaptive response of Seabuckthorn along an altitudinal gradient in trans-Himalaya

We measured leaf morphological characters in male and female *H.rhamnoides* individuals along an altitudinal gradient (2797-4117 m) in trans-Himalayan Ladakh region. Leaves become smaller in length and area, but became thicker with decreasing specific leaf area (SLA) with increasing altitude in both the gender. Leaf size, area, thickness, chlorophyll and petiole length were found to be higher in males than in females, while female had a higher SLA. When cuttings from the plants were grown in a common-garden experiment, the altitudinal effect disappeared for all morphological variables other than leaf thickness and SLA suggesting that most leaf morphological variation in *H. rhamnoides* is environmentally determined, but SLA and leaf thickness are also dependent on genotype. In the event of climate change, our study showed that phenotypic plasticity would be a crucial determinant of plant response in mountainous region. Effect of altitudinal gradient on leaf morphology was more conspicuous in males suggesting that males are more responsive to change in environmental conditions. Stressful environments will cause an added detrimental impact on females than on males. The results suggested that males will adapt better to the changing climate and may lead to a male-biased population in the event of climate change.

Gender-specific seasonal pattern and altitudinal variation in freeze tolerance responses in Seabuckthorn

In dioecious plants the adjustment to cold and freeze conditions may differ between male and female individuals. We measured the electrolyte leakage and proline contents in leaves and shoots in male and female SBT from mid August to mid December. A linear relation between electrolyte leakage and sampling period was observed in both male (R^2=0.871) and female (R^2=0.882) leaves. However, electrolyte leakage in shoot remained constant throughout the sampling period. Proline content in leaves showed a significant increasing trend from August to October followed by a steady decline from November onwards in both the gender. 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. Altitude (3202-3812 m amsl) of plant origin did not have a significant impact on electrolyte leakage in leaves and shoots. Increasing altitude 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). These results suggested sexually dissimilar responses to freezing in *H. rhamnoides* that male possess a better self protection mechanism than female. Leaves developed tolerance against frost more quickly than shoots when exposed to cold temperature.

SSR markers based genetic diversity characterization and marker-trait correlation for antioxidant properties in male and female populations of Seabuckthorn

Currently, most of commercial demands of SBT are fulfilled by extraction of the wild population causing reduction of its natural resources. Despite the recent studies using morphological and physiological traits, the genetic diversity targeting male and female populations at molecular level have not been studied. In this study, polymorphic SSR markers based evaluation of male and female populations (N: 180, six population) revealed high level of overall GD with higher GD in of male populations as compared to female populations, although not significant. The result suggested current ecological environment started causing selection pressure to females and the plant need attention for effective conservation of genetic resources. High gene flow, low genetic differentiation and high within population genetic variation suggest that most of the genetic diversity is restricted within population. Furthermore, neighbor joining and Bayesian-based STRUCTURE

analysis exhibited an intermixing in the clustering of male and female populations which might indicate the variation observed between them are controlled by autosomal genes in response to different environment and not sex-linked genes. Correlation of molecular data using Multiple Regression Analysis with SSR markers observed in significant correlation of the marker with phytochemical attributes in male and female populations. Current inferences on genetic diversity and population structure will allow in development of appropriate sampling strategies for optimization and implementation for conservation in *H. rhamnoides* genetic resources. Information generated from highly informative SSR marker in *H. rhamnoides* natural population revealed that the current study possibly be utilized to strategize the commercial cultivation, genetic improvement and implementation of selection of elite individual/ genotype in its major distribution site in India.

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