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Research Article

MINING THE PROTEIN REPERTOIRE OF A HIMALAYAN SHRUB, *HIPPOPHAE RHAMNOIDES* FOR ANTIFREEZE PROTEINS

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Abstract: Seabuckthorn (*Hippophae rhamnoides*), a cold desert shrub, can survive freezing temperature conditions and is considered as frost and drought tolerant. Earlier, antifreeze activity was reported in seabuckthorn seedlings grown under laboratory conditions. No reports are available on the cold hardiness of this huge bioresource available naturally in the Himalayan region. Detection of antifreeze activity in leaves and berries (splat assay and nanoliter osmometer) confirmed the presence of putative antifreeze proteins (AFPs) which may help in the survival of this plant under freezing conditions. Flower shaped ice crystals were observed in both leaves and berries while hexagonal ice crystals in seedlings indicated comparatively higher antifreeze activity in the wild parts. Splat assay results confirmed highest IRI activity in leaf (a 2.75 fold decrease in mean grain size of ice crystal after annealing) followed by berry (with 1.75 fold decrease) and least in the seedlings (with 1.5 fold decrease). Gel filtration chromatography resolved leaf fractions exhibited antifreeze activity in 34, 36 and 41 kDa while in berry fractions a 41 kDa polypeptide showed antifreeze activity. This is the first report showing presence of AFPs in (*H. rhamnoides*) leaf and berry. Shotgun proteomic analysis using Q-Exactive Orbitrap High Resolution Mass Spectrometer and functional annotation of leaf and berry proteins revealed their association with primary, secondary metabolism, defence/stress, redox regulation, signalling and structural remodelling majority of which are affected during cold stress. Further purification of these AFPs could open gates for commercial utilization of this plant growing abundantly in Himalayan regions of India.

Keywords: Antifreeze proteins; Ice recrystallisation inhibition; Splat assay; Nanoliter osmometer; Leaf; Berry.

Note: Coloured Figures and Supplementary Information available on Journal Website in "Archives" Section

Introduction

Freezing tolerant plants avoid freezing stress by formation of ice with the help of heterogeneous ice nucleators in their extracellular compartments thereby preventing lethal damage to inter and intracellular tissue (Pearce and Ashworth, 1992; Levitt, 1972). Further growth and propagation of extracellular ice to internal regions is constrained by various physiological and metabolic modifications during the cold acclimation like alterations in lipid composition, modification of cell wall (Wisniewski et al., 1991; 1993) changes in enzyme activities, (Olien, 1965), production of various antifreeze compounds, cryoprotective proteins and expression of antifreeze proteins (AFPs) (Griffith *et al*., 1992; Urrutia *et al*., 1992; Ewart *et al*., 1999; Yu and Griffith, 2001).

Antifreeze glycoproteins (AFGPs) were the first ice restricting proteins, discovered in Antarctic Notothenoid fish blood allowing survival of these fishes below the freezing point (-1.9 ºC) of their blood plasma (DeVries and Wohlschlang, 1969). AFPs include non-homologous, structurally diverse class of proteins present in wide range of organisms inhabiting the

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low temperature (LT) including polar fishes, fungi, bacteria (Duman and Olsen, 1993), insects, arthropods (Duman, 1979, Block and Duman, 1989) and plants (Griffith, 1992). Sequence and structural information is available for AFPs in fishes and from insects (*Choristoneura fumiferana* and *Dendroides canadensis*) (Duman *et al.,* 1991). Similar structural and phylogenetic analysis was done for 40 plant AFPs and based on the classification, these AFPs from plants are grouped into distinct families like leucine rich repeat (LRR), pathogenesis related (PR), hemagglutinin related (HR) and pleckstrin homology (PH) family (Muthukumaran *et al*., 2011). Recently, a novel non-proteinaceous antifreeze, Xylommanan antifreeze glycolipid capable of producing very high thermal hysteresis was purified from freeze-tolerant Alaskan beetle, *Upis ceramboides* (Walters *et al*., 2009). Similar TH-producing xylomannan glycolipid is present in evolutionary distinct taxa including plant (*Solanum dulcamara*), insects and frog (Walters *et al.,* 2011).

AFPs are ice adsorbing proteins which act by either preventing freezing or providing freezing tolerance. Thermal hysteresis (TH) and Ice recrystallisation inhibition (IRI) activity help in controlling the ice crystal size. Rather than preventing the growth of ice, AFPs modify the structure of ice or inhibit their growth and hence are also known as Ice Restructuring Proteins (IRPs) or Ice Recrystallisation Inhibition (IRI) Proteins (Venketesh and Dayananda, 2008). AFPs adsorb on non-basal planes of ice at the ice-water interface and exert a concentration-dependent effect on ice crystal growth morphology. These prevents accretion of water molecules to the growing crystal planes in a non-colligative manner to adsorp the interface which allows the formation of microcurvatures to depress the non equilibrium freezing point of a solution without affecting its melting point (March and Reisman, 1995; Antikainen and Griffith, 1997). The difference between the freezing and melting point is thermal hysteresis (DeVries, 1986; Davies and Hew, 1990). The phenomenon of TH was initially observed in larvae of the beetle *Tenebrio molitor*, which allowed water to supercool (Ramsay, 1964). AFPs possess ice binding domains which adsorb onto ice and irreversibly bind to these ice crystals thereby either modifying their shapes or inhibiting the growth. TH and IRI activity can be quantified using several methods as described (Sharma and Deswal, 2014). The most conventional ones are Splat assay and Nanoliter osmometer where presence or absence of antifreeze proteins can be analysed by observing the morphology of ice crystals. Ice crystals are hexagonal in the presence of AFPs, while they are disc shaped in their absence. Properties of AFPs differ in different organism which provide overwintering insect an advantage to survive the winter in a supercooled state as they exhibit higher thermal hysteresis (3 - 5 °C) than both fish and freezing-tolerant plants (0.1 - 3.5 °C).

AFPs were first reported in winter rye leaves in 1992 (Griffith, 1992; Urrutia *et al*., 1992). Various overwintering plant species (65) possess these proteins in different parts including stem, leaves, berries, roots and tubers (Doucet *et al*., 2000; Griffith and Yaish, 2004). Till date, AFPs have been purified from various low temperature inhabiting plants (Gupta and Deswal, 2014). In overwintering plants, ice recrystallization occurs commonly when these undergo freeze-thaw cycles at certain times of the year. Most of the AFPs are purified from the herbaceous plants and there are only few reports about AFPs from woody plants.

Seabuckthorn, a cold hardy shrub has the capability to withstand extreme temperature conditions and is considered frost and drought tolerant. In India, seabuckthorn resources can be found in colder regions of Himalayas including Lahaul and Spiti, Kinnaur and Chamba region of Himachal Pradesh, Ladakh in Jammu and Kashmir, Uttranchal, Sikkim and Arunachal Pradesh (Dwivedi, 2009, Singh 2003, 2006). Mostly the research is directed towards understanding the immunomodulatory, medicinal, radioprotective and therapeutic potential of seabuckthorn. Proteomics and genomics analyses of abiotic stress tolerance and the molecular mechanism involved in cold hardiness of this plant is poorly understood. Antifreeze proteins, chitinases (31 and 34 kDa) and Polygalacturonase inhibitor proteins (41 kDa) were purified from seedlings grown under laboratory conditions (Gupta and Deswal, 2012; 2014). Although

growing seedlings may save time, effort and money spent in collecting the material from its native places but whether there are quantitative and qualitative differences in naturally growing plants is not known. A detailed analysis and proteome studies of the plant growing under natural condition may provide a clearer picture about the freeze tolerance mechanism. Therefore, to determine whether and how these AFPs from seedlings differ from the ones present in the plants growing naturally, an initiative was taken to screen and partially purify the AFPs directly from leaves and berries of the plant collected from Lahaul and Spiti valley of Himachal Pradesh, India. This will also facilitate utilization of this valuable plant growing abundantly in Himalayan regions of India for commercial purposes. The material collected can be stored easily and used directly when required. Therefore, the objective of the study was to detect antifreeze activity in leaves and berries of *Hippophae rhamnoides* and to purify the putative AFPs. Owing to the exhibition of higher IRI activity, these AFPs could be used in different biotechnological and biomedical sectors like cryopreservation of frozen foods, cell lines, tissues and organs by preventing the freeze thaw induced changes in these. To the best of our knowledge presence of AFPs in wild parts of seabuckthorn are reported for the first time in this study.

Materials and methods

Plant material

Leaves and berries of *H. rhamnoides* were collected from Kaza along the river side of Rangrik village, 3100-3900 m above sea level (asl), Lahaul and Spiti valley of Himachal Pradesh, India (Figure 1). The tissue was washed with double distilled water to remove the dirt, frozen in liquid nitrogen and was transferred to the laboratory for storage at -80 °C. Pulp was obtained from berries by grinding the tissue in Tris-Cl (7.4), filtering the extract through double layered cheese cloth and storing it at -80 °C. Seedlings were obtained from seabuckthorn seeds isolated from *H. rhamnoides* berries. Seedlings were grown in growth chamber as previously described (Gupta and Deswal, 2012). Briefly, the seeds were plated on the germination paper to obtain uniform growth, incubated for 20 days in growth chamber at 24 ºC with 16 hr light/ 8 hr dark and a photosynthetic photon flux density (PPFD) of 270 mmol/m^2/s.

Protein extraction

Frozen tissue (Leaf and berry) was homogenised in the extraction buffer (1:1) containing 50 mM Tris pH-7.4, 20% glycerol, 2% 1 M Phenylmethylsulfonylfluoride (PMSF) and Polyvinylpolypyrolidine (PVPP). The slurry obtained was centrifuged at 12,500 rpm, 4 °C for 25 mins. The debris was discarded and the supernatant was acetone precipitated overnight at -20 °C. The pellet containing the proteins was obtained by centrifugation at 10,000 g for 10 min, air dried and dissolved in 50 mM Tris-HCl pH 7.4. Protein concentration was determined according to Bradford (1976) with bovine serum albumin (BSA, Sigma) as a standard.

Gel filtration (G-100) chromatography

Sephadex G-100 column (7cm × 2.5cm), equilibrated with 50mM Tris-HCl, pH 7.4 was packed at 4 ml/min and homogeneity of the column was checked using Blue Dextran dye. Crude protein extract (15 mg) was loaded on the gel filtration column and protein fractions of 5 ml were eluted (at 1 ml/min) using 50mM Tris-HCl, pH 7.4 and 150 mM NaCl. Absorbance (280 nm) was taken using spectrophotometer to measure the protein concentration. The protein fractions were resolved on SDS-PAGE and their antifreeze activity was measured using Nanoliter osmometer and Splat assay.

Protein profile using SDS-PAGE

SDS-PAGE was performed on a 10% polyacrylamide gel (Amersham) according to Laemmli (1970). Molecular marker (GE Healthcare, India) was the standard protein mixture consisting of phosphorylase b (97.0 kDa), BSA (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14.4 kDa). The protein samples obtained were loaded on the gel after boiling these in sample buffer (0.5 M Tris-HCL pH 6.8, 10% glycerol, 10% SDS, 1% Bromophenol blue and β-mercaptoethanol) and electrophoresis was performed at 80 V for stacking gel and 120 V for

separating gel, to obtain the protein profile. After electrophoresis, gels were silver stained (Merril *et al*., 1983).

Detection of antifreeze activity by Nanoliter osmometer (Ice crystal morphology) and Splat assay (IRI activity)

Nanoliter osmometer and Splat assay are two commonly used techniques for the measurement of antifreeze activity. IRI activity can be quantified using Splat assay and the presence or absence of antifreeze proteins can be analysed by observing the morphology of ice crystals. Moreover, serial dilutions can be prepared to determine the concentration below which RI activity is no longer detected, defined as endpoint of IRI. For Modified Sucrose Sandwich Splat Assay (Smallwood *et al*., 1999; Tomczack *et al*., 2003), proteins were dissolved in 30% (w/v) sucrose in 20 mM ammonium bicarbonate (ABC) and 2 µl sample was sandwiched between two round coverslips, snap frozen in heptanes kept at - 80 °C and then transferred to a glass viewing chamber maintained at - 6 °C by temperature controlled water bath, containing ethylene glycol as a coolant. For the quantification of the ice recrystallisation inhibition (IRI) activity, the diameters of the ice crystals in each field after annealing, were measured using Image J software and the end point of RI was calculated. Three different fields of an image were chosen and the diameter of ice crystals was calculated in each field in triplicates. The ice crystals diameters and the corresponding Ice Recrystallisation Inhibition (IRI) percentage of protein fractions were compared with 50 mM Tris buffer, pH 7.4, taken as control. Average and standard deviation of the replicates were calculated to find the percent IRI of each fraction.

Ice crystals are hexagonal in the presence of AFPs whereas these are disc shaped in their absence. Nanoliter osmometer coupled with cryostage fitted with phase contrast microscope (Nikon eclipse) was used to observe the ice crystal morphology of protein extracts to determine the presence or absence of AFPs. In brief, prior to sample loading the disc is loaded with heavy mineral oil at the base of the well to allow loading of the sample. Protein sample (20 nl) was loaded using Hamilton syringe and flash frozen at -20 °C to form a population of ice crystals. The temperature was gradually increased to allow melting of the ice crystals until a single ice crystal is left. AFPs were allowed to bind the ice crystal and the temperature was further decreased to observe the modified ice crystal morphology such that ice crystals are hexagon or flower shaped in their presence due to local ice surface curvature effects promoted by adsorption-inhibition mechanism of AFPS on ice-solution boundary (DeVries, 1986). To reaffirm that the activity was due to the antifreeze proteins present in the sample, the samples were treated overnight with Proteinase-K (1 mg/ml) and the results were verified by the loss of activity and appearance of disc shaped ice crystals after the treatment. 20 mM ABC (buffer) was used as negative control. All precautions were taken to avoid non specific antifreeze activity therefore the disc was kept overnight in organic solvent, sonicated for 5 mins and vacuum dried to remove any contamination left in the well which could otherwise give false positive antifreeze activity.

Statistical analysis

Student t-test was performed to observe the significant changes ($p < 0.05$) between the readings of control sample and different protein fractions. All the experiments and assays were performed in triplicates and representative images are shown.

In-solution digestion, shotgun proteomic analysis and functional annotation

For identification of the complete proteome, proteins (100 µg) isolated from *H. rhamnoides* leaves and berries were subjected to in-solution trypsin digestion (Gundry *et al.,* 2009). Peptides obtained, were dissolved in mobile phase A (water/acetonitrile, 98:2 v/v; 0.1% formic acid). Further desalting of tryptic peptides was performed by reversed-phase chromatography using an ultra high performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific, USA) equipped with a Acclaim PepMap 100 trap column (100 µm × 2 cm, nanoViper C18, 5 µm). The samples were subsequently washed with 98% mobile phase A

at 6 µL/min,6 mins and were separated on a capillary column (75 ìm × 15 cm, nanoViper C18, 3 µm,) at 400 nL/min. The Liquid chromatography (LC) analytical gradient was run at 2% to 35% mobile phase B over 90 min, then 35% to 95%, 10 min, followed by 90%, 5 min and finally 5% for 15 min. Resulting peptides were electro-sprayed through a coated silica tip at an ion spray voltage of 2,000 eV. The Ultra high performance liquid chromatography (UHPLC) was coupled with a heated electrospray ionization source (HESI-II) to the quadrupole-based mass spectrometer Q ExactiveTM Orbitrap High Resolution Mass Spectrometer (Thermo Fisher Scientific, USA). The MS spectra were acquired at a resolution of 70,000 (200 m/z) in a mass range of 350 -1,800 m/z. A maximum injection time was set to 100 ms for ion accumulation. Eluted samples were used for MS/MS events, measured in a data dependent mode for the 10 most abundant peaks, in the high mass accuracy Orbitrap after ion activation/ dissociation with Higher Energy C-trap Dissociation (HCD) at 27 collision energy in a 100-1650 m/z mass range. Raw proteomic data was searched against Viridiplantae protein database obtained from NCBInr (National Center for Biotechnology Information, non redundant) using Proteome Discoverer 1.4 (Thermo Fisher Scientific, USA) software and SEQUEST as a search engine. (Supplementary Information, Tables 1 & 2). Several filtering criterion for obtaining significant scores including Xcorr > 2, indicative of a good correlation, unique peptides and matched ion count were used to isolate correct identifications from random assignments. Functional annotations of the identified proteins were carried out using Gene Ontology (GO) database (http://www.geneontology.org).

Results and Discussion

Leaf extract showed maximum antifreeze activity

A clear protein profile for seedling, leaf and berry was obtained using Tris-glycerol extraction (Figure 2 upper panel). The gel profile corresponded with the estimated protein concentration (Bradford assay) showing higher concentration of proteins in the seedling extract (0.78 mg/ml) followed by leaf (0.3753 mg/ml) and (0.2183 mg/ml) berry. The protein profile showed 37 polypeptides (114 -14 kDa) in seedling, 31 polypeptides (104 -10 kDa) in leaf and 24 (104 -10 kDa) polypeptides in berry. To confirm the presence of AFPs in wild parts, their antifreeze activity was analyzed.

In splat assay, snap freezing formed many ice crystals and on raising the temperature, the mean ice crystal size increased due to irruptive recrystallisation, this ice recrystallisation is impeded in the presence of AFPs (Yeh and Feeney, 1996). Bright Field images using Stereoscopic zoom microscope (Zeiss) showed higher IRI activity in the crude leaf exhibiting 2.75 fold decrease in the mean grain size followed by

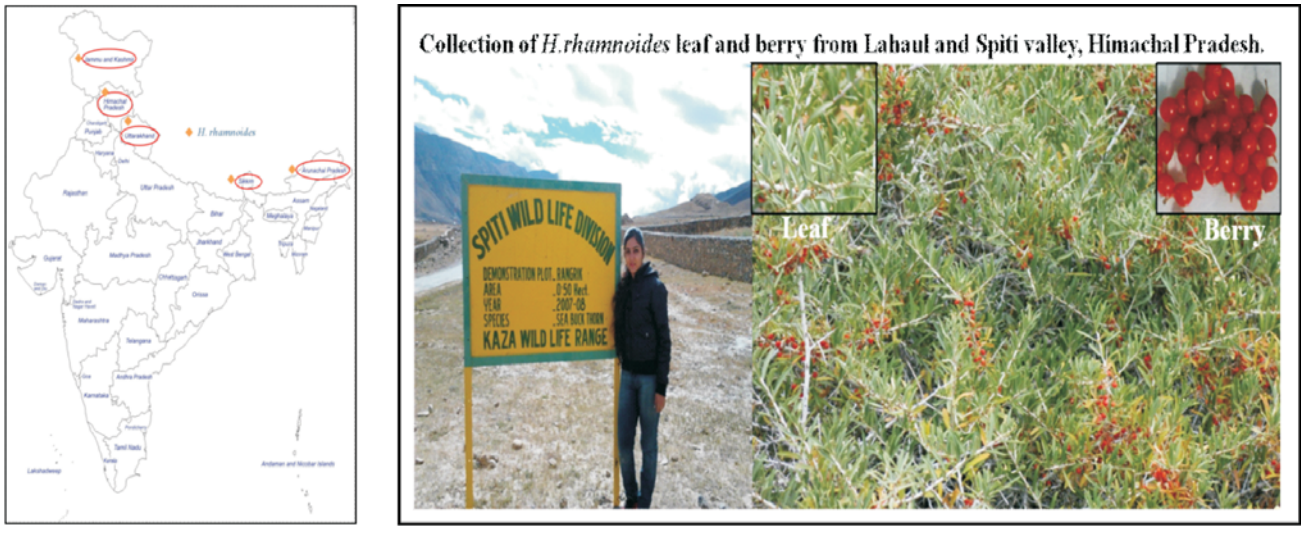

Figure 1 A): **Illustrative map showing distribution of *Hippophae rhamnoides* in India.** B) **The site of *H.rhamnoides* leaf and berry collection along the river side of Rangrik village (3100-3900 m asl), Lahaul and Spiti valley, Himachal Pradesh.**

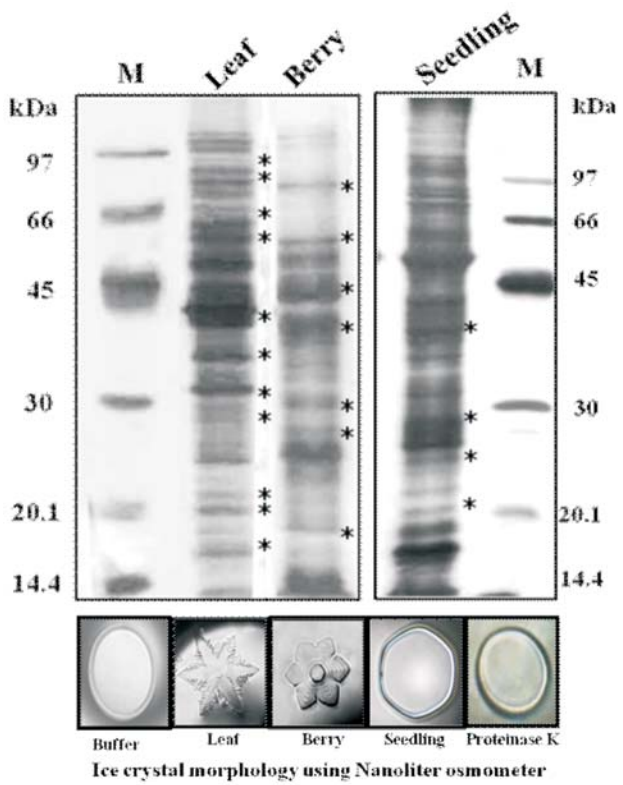

Figure 2: **Silver stained SDS-PAGE gels (10 %, upper panel) showing the protein profile of *H.rhamnoides* leaf, berry and seedling fractions. Ice crystal morphology using Nanoliter osmometer (lower panel) to measure the antifreeze activity. The flower (leaf, berry) and hexagon shaped (seedling) ice crystals confirmed the presence of antifreeze activity in Seabuckthorn (0.2 mg/ml) while disc shaped ice crystals were observed in negative control (buffer).**

berry where 1.75 fold decrease was observed and least in seedling showing 1.5 fold decrease in the grain size at equal protein concentration (0.2 mg/ml) in comparison with the negative control (Buffer) suggesting that the wild parts possess higher IRI activity than the laboratory grown seedlings. Endpoint of IRI activity, the concentration below which RI activity is no longer detected was found to be 25 µg/ml for leaf and 50 µg/ml for berry. *Daucus carota* crude root extract showed IRI activity till 150 µg/ml while the 36 kDa purified protein, PGIP showed IRI activity even at as low as < 1 µg/ml (Smallwood *et al*., 1999). Similiarly, in apoplast extract of Japanese radish tuber and leaf IRI endpoint was determined as 46 and 16 µg/ml respectively (Kawahara *et al*., 2009). In Forsythia, a 20 kDa dehydrin showed IRI activity at 6 µg/ml (Simpson *et al*., 2005). In *H.rhamnoides* shoot apoplast the purified AFPs PGIP showed endpoint at 12 µg/ml, whereas chitinase (31 and 34 kDa) at 60 and 120 µg/ml (Gupta and Deswal, 2012; 2014). The results suggest the activity of crude leaf and berry AFPs are in the intermediate range and once the proteins are purified, more accurate and specific details may be obtained.

Lower IRI activity was observed in G-100 column eluted leaf and berry fractions relative to the crude extract due to separation and distribution of activity in different fractions. Ice crystal morphology obtained using Nanoliter osmometer (Otago osmometer, NewZealand) also confirmed higher antifreeze activity in leaf and berry, represented by the presence of characteristic flower shaped ice crystals as compared to seedling where hexagonal ice crystals were observed indicating weak activity than the wild parts. The ice crystals were disc shaped in the negative control. Moreover, absence of activity after proteinase K treatment confirmed the presence of antifreeze activity due to AFPs (Figure 2 lower panel). Similiar flower shaped ice crystals have been observed in recombinant cold-induced purified AFPs glucanases (34 kDa) from winter rye (Yaish *et al.,* 2006) and chitinases (34 & 31 kDa) from *H. rhamnoides* seedlings (Gupta and Deswal, 2014) owing to the preferential binding of AFPs to secondary prism planes of ice. An attempt was made to resolve the proteins by gel filtration chromatography to obtain activity enriched fractions.

Polypeptides of 34, 36, and 41 kDa showed antifreeze activity in Leaf

Proteins (15 mg) extracted from leaf were loaded on the Sephadex (G-100) size exclusion column to segregate the proteins according to their size and collect the putative AFPs enriched fractions showing higher antifreeze activity for further analysis. Fractions of 5 ml were eluted at a flow rate of 1 ml/min and their absorbance was taken at 280 nm (Figure 3). The readings obtained were found consistent with the SDS-PAGE profile of these fractions and polypeptides of 104-10 kDa were observed. The efforts to purify individual antifreeze proteins using the conventional size exclusion chromatography were unsuccessful and all the major polypeptides co-eluted in three non-

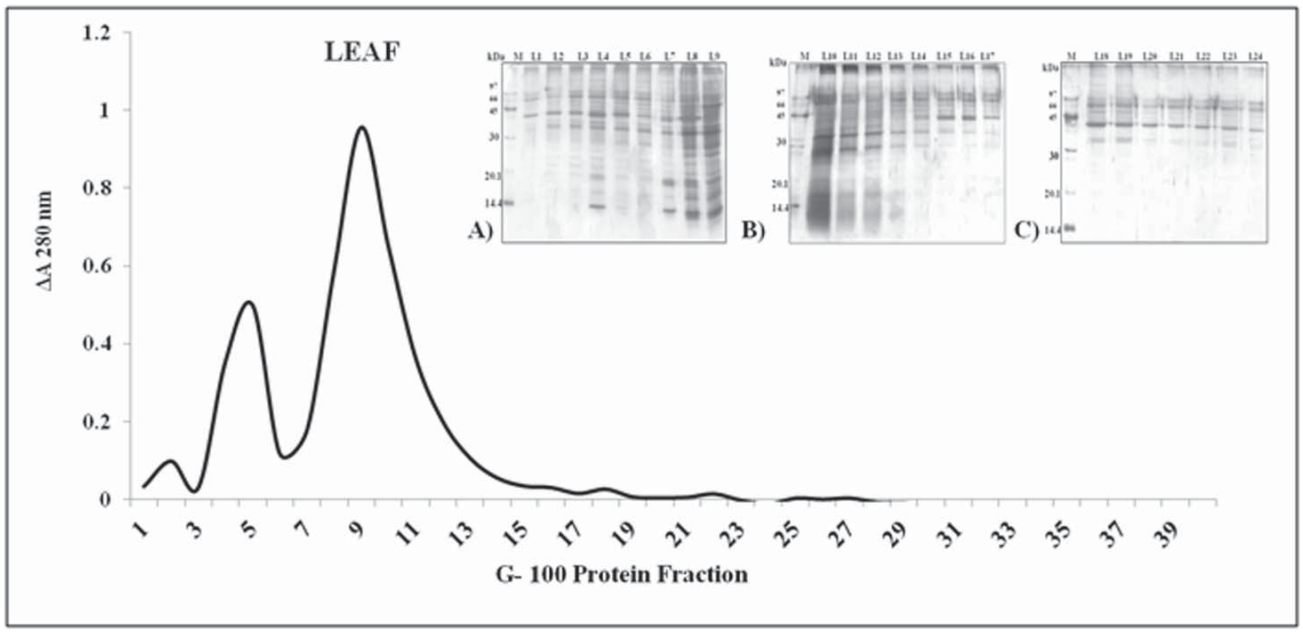

Figure 3: **Absorbance (280 nm) of leaf fractions resolved after Sephadex G- 100 column chromatography.** (A - C inset) **Silver stained SDS-PAGE gels showing the protein profile of leaf fractions eluted from Sephadex G-100 column.**

overlapping peaks. Polypeptides of higher molecular weight (66-30 kDa) were common in all the fractions, while lower molecular weight polypeptides (30-16 kDa) were observed in fractions 4 to 13 only (Figure 3 inset).

Splat assay and corresponding IRI activity showed high IRI activity in fractions 6, 7, 8, 10, 15, 16, 17, 18, 20 and 22 (Figure 4A). Maximum IRI activity (52.4%) was observed in fraction 15. The corresponding gel profile showed that polypeptides of 34, 36 and 41 kDa were present in all the active fractions suggesting these to be strongly associated with the higher antifreeze activity (Figure 4B upper panel) while polypeptides of 30-14 kDa did not contribute significantly to the overall activity as even the fractions (6, 7, 15, 18, 20 and 22) without these polypeptides exhibited higher IRI activity (Figure 4C). Similarly, ice crystal morphology of active leaf fractions revealed flower shaped ice crystal in fraction 15 and 18 confirming higher antifreeze activity whereas fractions 6,10 and 22 (47.96% and 41%) showed hexagonal structure suggesting moderate antifreeze activity (Figure 4B lower panel). It can be concluded that 34, 36, and 41 kDa polypeptides may be the putative antifreeze proteins in partially purified leaf extract.

A 41 kDa polypeptide is associated with antifreeze activity in Berry

Proteins (15 mg) extracted from berry pulp as mentioned in materials and methods were loaded on the Sephadex (G-100) gel filtration column for fractionation of putative AFPs. The absorbance and elution profile (Figure 5) obtained were consistent with the protein pattern on SDS- PAGE of these fractions. Polypeptides (27) of 104-10 kDa were obtained in G-100 column eluted berry protein fractions. Higher molecular weight polypeptides of 97- 41 kDa were observed in all the fractions (1-24) while low molecular weight 10 kDa was observed exclusively in fractions 13 to 19.

IRI analysis using splat assay showed marked IRI activity in berry protein fractions (Figure 6A) as observed by a little change in size of ice crystals of berry protein fractions even after annealing. High IRI activity was observed in fractions 10, 12, 18, 21, 22 and 24 and maximum activity (38.09 %) was shown by fraction no. 18 (Figure 6B). It was observed that although the overall IRI activity is higher in the presence of multiple active polypeptides together in the corresponding fraction 18 but the major polypeptide involved in providing activity is 41 kDa and all the active fractions containing this single protein showed

change in ice crystal morphology to hexagon (fraction 10, 12 and 22). The results obtained by Nanoliter osmometer were in accordance with the data obtained from antifreeze activity. Flower shaped ice crystal was observed in fraction no. 18 indicative of highest antifreeze activity while fractions 10 and 12 showed a weak hexagonal structure suggesting moderate activity in these fractions. In fraction 22 and 24 the ice crystals were hexagonal in shape suggesting that polypeptide of 41 kDa is solely associated with high antifreeze activity (Figure 6B, 6C).

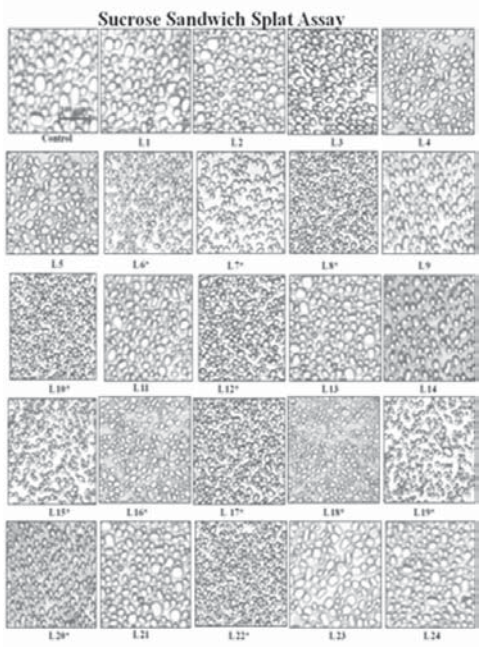

Figure 4a

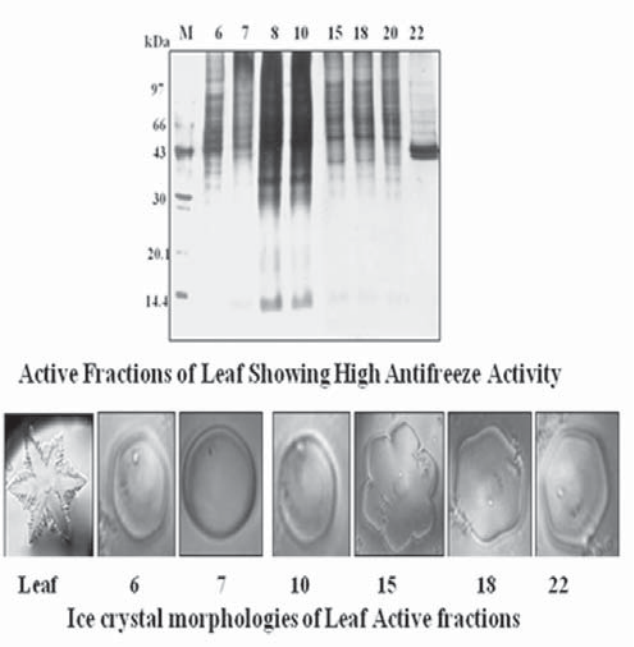

Figure 4b

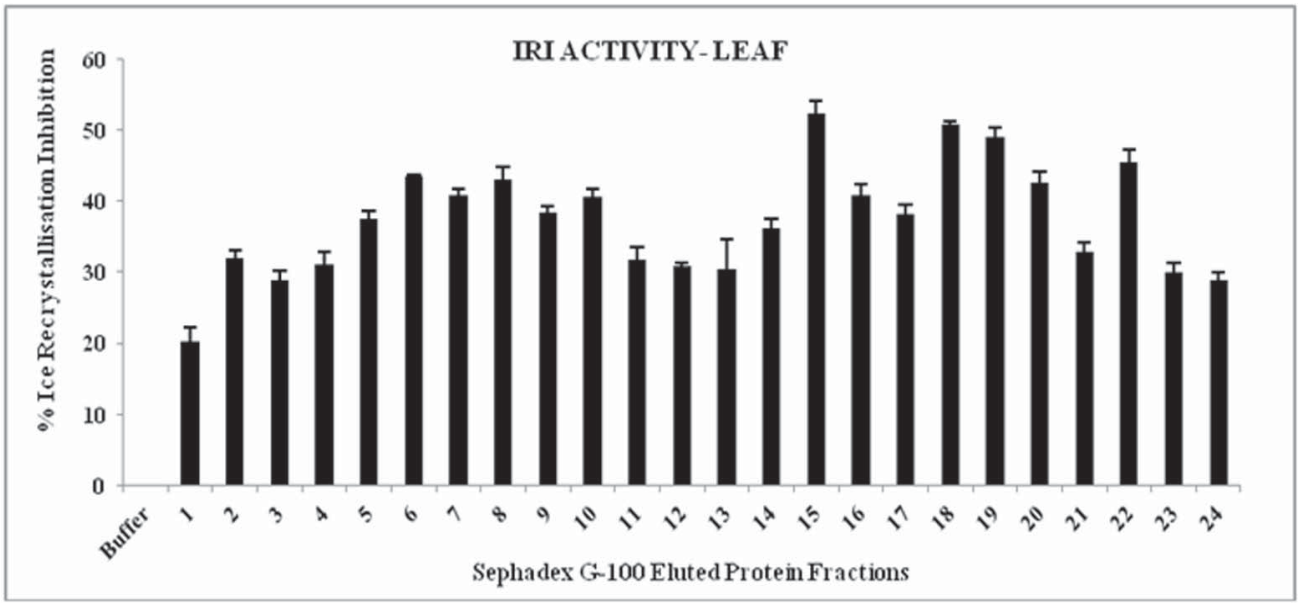

Figure 4: **A) IRI assays, for analysis of antifreeze activity in seabuckthorn leaf in control and G-100 column eluted protein fractions of leaf. B) Silver stained (10 %) SDS-PAGE gel (upper panel) showing protein profile of leaf fractions with high IRI activity. Ice crystal morphologies (lower panel) showing hexagon and flower shaped crystals confirming presence of putative antifreeze polypeptides in leaf active fractions. C) Quantification of IRI activity in G-100 eluted leaf protein fractions. Significant readings ($p < 0.05$) calculated by student's T-test.**

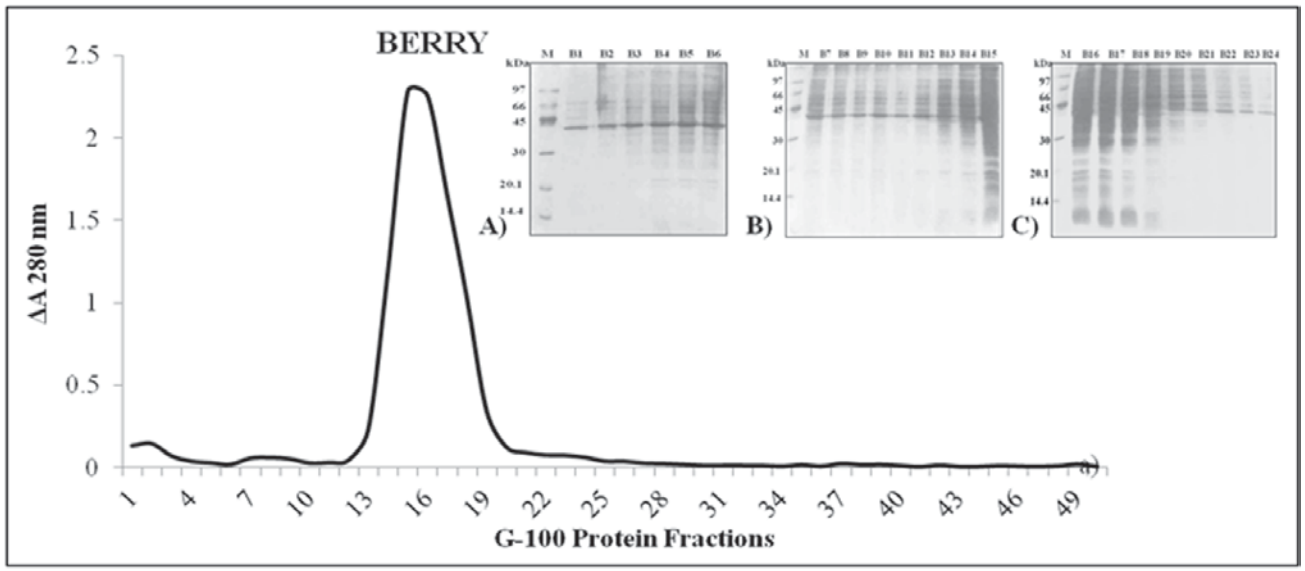

Figure 5 : **Absorbance (280 nm) of berry fractions resolved after Sephadex G- 100 column.** (A - C inset) **Silver stained SDS-PAGE gels showing the protein profile of berry fractions.**

Functional annotation revealed proteins common to metabolic and stress responsive pathways

LT induced secretome analysis revealed the regulation of various proteins during cold stress and NanoLC-MS/MS identification revealed the involvement of these proteins in redox regulation, defence, stress, signalling, and metabolism in seedlings under LT stress. Cold induced differential abundance of putative AFPs like thaumatin-like proteins and chitinases were also observed during freezing stress. Therefore, identification of the complete proteome from *H. rhamnoides* leaves and berries was attempted

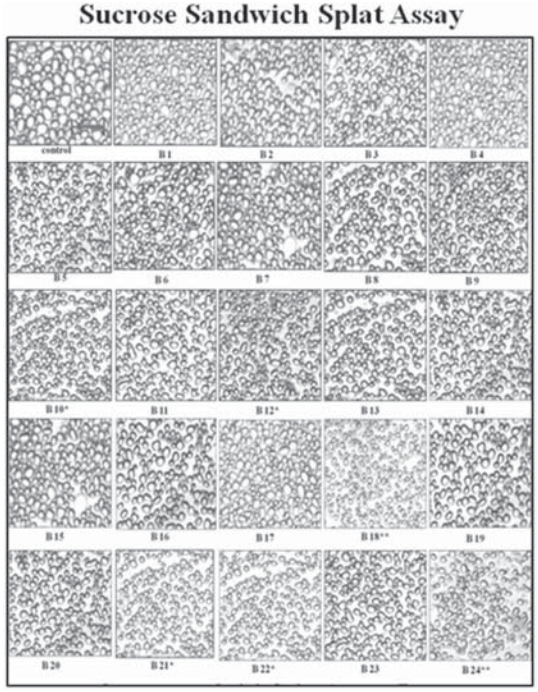

Figure 6a

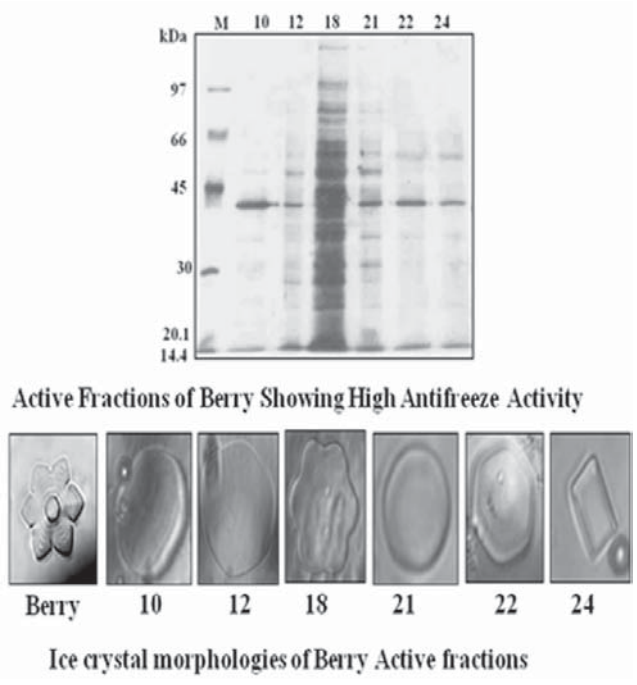

Figure 6b

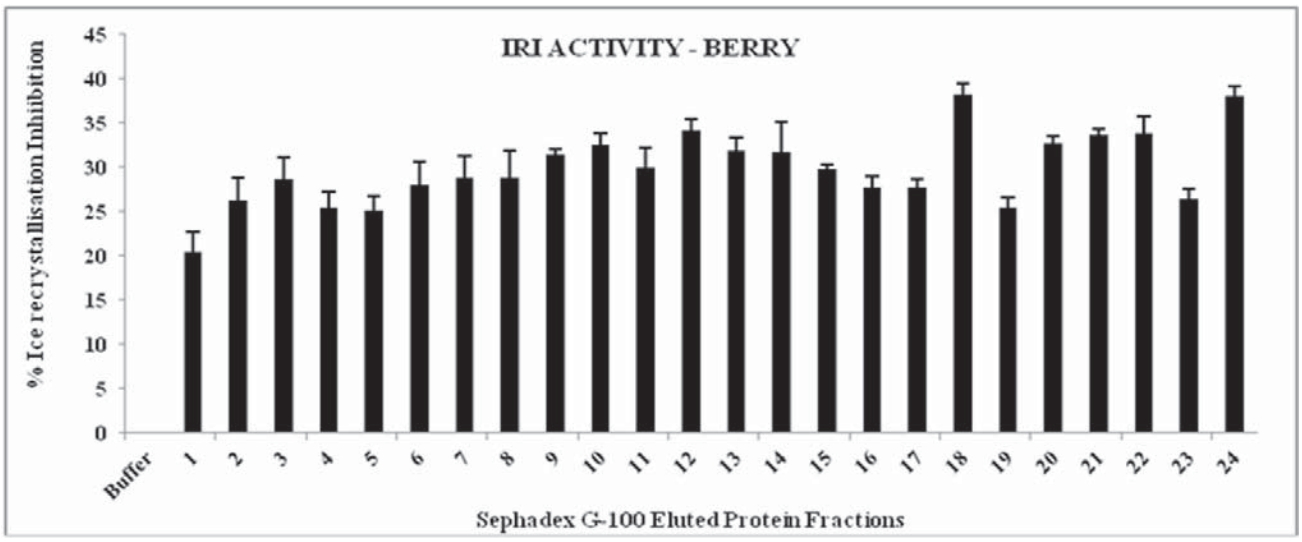

Figure 6: **A) Splat assay, for analysis of antifreeze activity in G-100 column eluted Berry fractions. B) Silver stained (10 %) SDS-PAGE gel (upper panel) showing the protein profile of Berry fractions with high IRI activity. Modified ice crystal growth (Hexagon and flower shaped) in Berry fractions (lower panel) confirming presence of putative antifreeze polypeptides. C) Quantification of IRI activity in G-100 eluted berry protein fractions.**

using shotgun proteomic analysis to provide better understanding of the mechanism involved in the cold hardiness of this plant.

Functional categorization of the identified proteins was done using GO software (Supplementary information). Q-Exactive Orbitrap High Resolution Mass Spectrometer based shotgun proteomic analysis which led to the identification of 397 proteins from leaf and berry. Lesser number of proteins identification may be due to the unavailability of the genome database of seabukthorn (Tables 1 and 2). Moreover, not even a single plant from the Elaeagnaceae or its phylogenetic related family has been sequenced, making protein identification from seabuckthorn more difficult. In leaf, the proteins were broadly clustered into 15 functional categories while 7 categories were found in berry (Figure 7). Proteins associated with primary metabolism, photosynthesis, unknown, energy metabolism, others, protein folding and protein degradation were common in both leaf and berry. However, additional proteins related with signaling, stress/defense, transport, structure, cell division, secondary metabolism, redox regulation and protein synthesis were found exclusively in leaf. These results suggest active energy metabolic processes in the leaf and berry as majority of the proteins belong to these categories. Photosynthetic machinery related proteins including ATP synthase subunits, photosystem II protein, cytochrome, RuBisCO subunit-binding protein, chlorophyll a-b binding protein, sedoheptulose-1,7-bisphosphatase and photosynthetic electron transfer chain were identified. The presence of proteins involved in structural modifications and transport regulation suggested the translocation of nutrients and restructuring during the growth, stress and senescence of the wild parts. Regulation of various cold stress-mediated physiological features, metabolites and their roles during cold-stress networks are well reported in literature (Seki *et al*., 2002; Janska, *et al*., 2010; Zhang *et al*., 2014). Cold acclimation induces accumulation of reactive oxygen species (ROS), which acts as a signaling molecule in mediating important signal transduction pathways under temperature stress and leads to the reprogramming of the transcriptome and proteome through Ca^{2+} signaling, activation of protein kinases, redox-responsive transcription factors and regulation of primary and secondary metabolites. The presence of various receptor kinases, calmodulin and a variety of enzymatic and non-enzymatic antioxidants (superoxide dismutase, glutathione peroxidase, glutathione reductase, ascorbate peroxidase and catalase) suggested an active signalling during biotic and abiotic stress and their role in maintenance of cellular redox

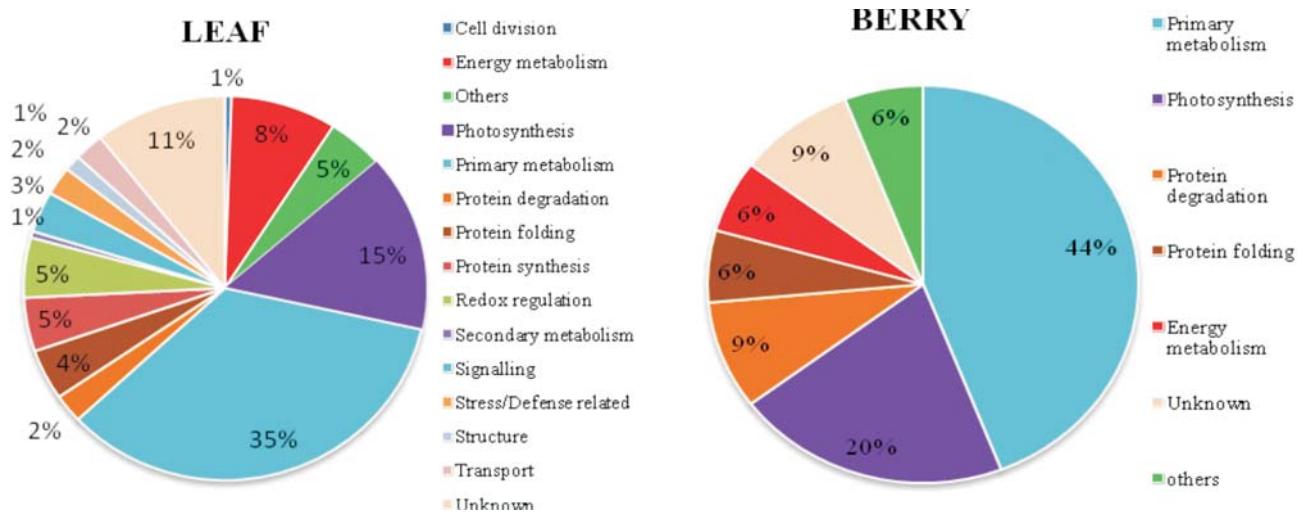

Figure 7: **Gene ontology based functional annotation of the leaf and berry proteins identified using shotgun proteomic approach.**

homeostasis leading to the activation of down regulated genes and proteins associated with stress responses to optimize defense and acclimation of plant (Chen and Li, 2001). Disease resistance protein (RPP8), a LRR protein identified in leaf triggers a defense system including the hypersensitive response to restrict the pathogen growth. Various LRR-domain containing AFPs are reported in literature (Meyer *et al*., 1999) thereby suggesting the putative presence of dual functioning PR proteins in seabuckthorn.

Purification of AFPs & homology with other protein families

AFPs have been purified from leaves and berries of various overwintering plants suggesting expression of distinct families of AFPs like cold-induced chitinases (24.79 and 47.77 kDa) in *Annona cherimola* berry (Goni *et al*., 2013), agglutinins (34.7 and 37.1 kDa) in *Ammopiptanthus mongolicus* leaf (Wang *et al*., 2003), dehydrins (20 kDa) in *Forsythia suspense* (Simpson *et al*., 2005), PR proteins (15 - 38 kDa) and thaumatins (16 and 25 kDa) in winter rye (Hon *et al*., 1995).

Literature studies have shown the dual functionality of AFPs in various cold tolerant plants. Full-length nucleotide sequence of genes encoding AFPs from plants like *Ammopiptanthus mongolicus*, Bittersweet nightshade, Peach and winter rye have suggested their homology to pathogenesis-related (PR) proteins (Hon *et al*., 1995; Huang *et al*., 2001; Fei *et al*., 1994; 2001). Moreover, out of all the AFPs identified till date, approximately 60% of these show dual functionality possessing both antifreeze and antifungal, hydrolytic activity. This helps the plant to exhibit both cold tolerance and disease resistance against psychrophilic pathogens at subzero temperatures.

Although the mechanism for the switch between the antifreeze and hydrolytic activity is still not very clear. However, the possibility is some PTMs and remodelling/restructuring of proteins may be involved (Griffith and Yaish, 2004). Calcium is shown to regulate the hydrolytic and antifreeze activities of AFPs (chitinases) in *H.rhamnoides* by changing their secondary structure (Gupta and Deswal, 2014).

In leaf 34, 36 and 41 kDa were the major polypeptides shown to be strongly associated with high antifreeze activity whereas in berry polypeptide of 41 kDa exhibit high antifreeze activity. In *H. rhamnoides* shoot apoplast a 41 kDa PGIP and two chitinases of 34 and 31 kDa were purified. Further efforts to purify the similar dual functioning AFPs using colloidal chitin affinity chromatography led to the purification of 31 and 34 kDa chitinases in leaf and 24 and 10 kDa in berry confirming the presence of dual functioning PR proteins (chitinases) in the wild grown seabuckthorn tissues (Sharma and Deswal, 2015 In Press). All these observations suggested the

presence of dual functioning AFPs in both leaf and berry of seabuckthorn. However, further purification and characterization of these AFPs is required for a deeper understanding of their dual functionality.

Conclusions

Presence of antifreeze activity was confirmed in both leaf and berry of *H. rhamnoides*. The major proteins associated with the antifreeze activity in leaf were 34, 36 and 41 kDa whereas in berry it was 41 kDa. Splat assay and Nanoliter osmometer results confirmed that active fractions possessing these peptides possess high ice recrystallisation inhibition activity and modified the ice crystal growth to hexagon or flower shaped. AFPs showing homology with different pathogenesis-related proteins suggest multiple roles of these proteins in plant. Shotgun proteomic analysis revealed the complete repertoire of proteins, with majority of the proteins involved in metabolic processes, structure remodelling, signalling and few dual functioning stress related PR proteins and majority of these proteins are regulated during the stress signalling pathways. Further characterisation and confirmation of the above predictions could provide a better scenario of antifreeze proteins in seabuckthorn. The future work would be focused on the purification and further validation of these AFPs from leaves and berries samples and their utilization for biotechnological applications.

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Abbreviations

AFPs, Antifreeze proteins; AFGPs, Antifreeze glycoproteins; LT, Low Temperature; LRR, leucine rich repeat; PR, Pathogenesis related; HR, Hemagglutinin related; PH, Pleckstrin homology; TH, Thermal Hysteresis; IRI, Ice recrystallisation inhibition; asl, Above sea level; PPFD, Photosynthetic photon flux density; PMSF, Phenylmethylsulfonylfluoride; PVPP, Polyvinylpolypyrolidine; BSA, Bovine serum albumin; ABC, Ammonium bicarbonate; UPLC, Ultra high performance liquid chromatography; LC, Liquid chromatography; MS, Mass spectrometry; HES, Heated electrospray ionization source; HCD, Higher Energy C-trap Dissociation; NCBI, National Center for Biotechnology Information ROS, Reactive oxygen species; GO, Gene Ontology; RBCs, Red blood cells.

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