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Resurrection of the Fern "*Cheilanthes albomarginata* Clarke" Involves Utilization of Trehalose as Energy Source and Accumulation of Protective Stress Metabolites

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Research article

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Abstract

Most plants are unable to survive desiccation to an air-dried state, but a small group of plants known as resurrection plants can tolerate extreme desiccation, and regain normal function after rehydration. *Cheilanthes albomarginata* is one such resurrection fern, which has been analyzed in this study for metabolite changes during resurrection. In this study, we have tested *C. albomarginata* fronds from ten different geographical regions of Himachal Pradesh for the presence of trehalose, proline, and glycine betaine. The profile of trehalose sugar during resurrection was investigated using chromatographic techniques and enzymatic assays. Analysis of extracted sugars by High performance liquid chromatography (HPLC) showed that trehalose levels were highest in dehydrated stage of *C. albomarginata* (46 %), which reduced to 28% upon rehydration. Fourier Transform InfraRed spectroscopy (FTIR) analysis of intracellular sugars in crude extracts of *C. albomarginata* indicated the closest match of the sugar in the samples with trehalose rather than sucrose. Quantitative estimation of extracted trehalose sugar of *C. albomarginata* using crude trehalase further confirmed the utilization of trehalose during resurrection. Thus, trehalose appears to be the major sugar accumulating during dehydrated condition, with an active role in resurrection of *C. albomarginata*. Proline and Glycine betaine were observed to accumulate during dehydration stage in *C. albomarginata*, and remained largely unaltered during rehydrated state, indicating their protective role during resurrection.

Keywords: Resurrection; *Cheilanthes albomarginata*; Ferns; Trehalose; Proline; Glycine betaine

Abbreviations: HPLC: High Performance Liquid Chromatography; FTIR: Fourier Transform InfraRed Spectroscopy; TLC: Thin Layer Chromatography; MCT: Mercury Cadmium Telluride; GB: Glycine-Betaine; RWC: Relative Water Content; LSD: Least Significant Difference; DT: Desiccation Tolerance.

Introduction

Elevation in the emissions of greenhouse gases, increased world population and worldwide climate change are some of the major key issues from the last few decades [1-3]. The ultimate result of these issues is global water shortage which now is one of the another major challenge, affecting livelihood and agriculture worldwide to larger extent [4]. Due to the global water shortage, sufficient amount of water is not available to the every corner of world and as a result, drought has become a major threat from the last few decades to agriculture globally as water scarcity contributes to the highest percentage death rate of many important crops. To feed every person in this planet, the requirements for valuable crops with high productivity are needed. But during heavy drought, most of plants do not survive and dies out. Exceptionally, a few plants known as resurrection plants, can survive in these extreme conditions [5]. Thus, from the few last decades, lots of efforts have been made by many researchers to understand the physiology, development and life cycle of these plants at cellular, biochemical and molecular level.

Resurrection plants are characterized by the ability to revive or becoming alive after seeming to be dead. Upon dehydration, resurrection plants shrivel up and fold their leaves, until water is available [6-8]. Thus, it is important to understand the mechanism that how these plants adapt to the water stress and how they survive during the extreme drought. Since metabolites play a key role during drought stress and revival upon rehydration [9]. Therefore, analysis of metabolomics of these plants can help the researchers to find out the mysteries behind the survival in the extreme conditions.

Cheilanthes albomarginata, a resurrection fern undergo its dehydration and rehydration cyles according to the environmental conditions [10]. Thus vegetative desiccation tolerance in this fern is seasonally regulated. The leaves of *C. albomarginata* culred up during summer season and revived again when water is available. The RWC values in this fern showed that fern is able to survive even in 8% of remaining water and can also able to recover the chlorophyll content and metabolic activity at full rehydration. Many physiological factors such as light, temperature (data not published), water and developmental stage appear to have an enhancement effect on the resurrection activity of *C. albomarginata* fronds [10].

In this study, an effort has been made to understand the metabolomics involved during the resurrection using various techniques and assays. The samples of *C. albomarginata* were collected and analysed during resurrection. The fresh stage of sample is considered as primary control and purified standards as secondary control for comparing and analyzing the metabolic changes in dry and rehydrated stages. Finally, the correlation statistics is applied on the results obtained in each analysis with relative water content, time for complete resurrection and altitude. To our knowledge, this is the first study to provide insight into the resurrection activity in *C. albomarginata* and its metabolic characterization.

Materials and Methods

Collection and Identification of Fern Samples

The fronds of C. *albomarginata* fern were collected during rainy season (July-August) from Solan and adjoining areas, Himachal Pradesh, India [10]. The fronds were first washed with 1% (v/v) H_2O_2 and then with distilled water and used immediately upon collection. The remaining plants were stored at -80 °C. All samples were identified from herbarium of Punjab University, Chandigarh India, University of Horticulture and Forestry, Nauni, Solan, H.P., and other resources [11].

Thin Layer Chromatography (TLC) Analysis of Sugars during Resurrection of *C. albomarginata*

Ethanolic crude extracts of powdered fronds of *C. albomarginata* were prepared, weighed and re-suspended in ethanol to a concentration of 1 mg/ mL, and used for TLC analysis of sugars. For the analysis of sugars, standards of trehalose (Sigma-Aldrich, USA) and glucose (Himedia Labs, Mumbai) were spotted along with ethanolic extracts (1 mg/ mL) of fronds of fresh, dry and rehydrated stages of *C. albomarginata* onto pre-cast silica gel TLC plates (Merck, Millipore India Pvt. Ltd., Bangalore) and developed with a solvent system containing butanol : pyridine : water (in a ratio of 15 : 30 : 20, v/v) as the mobile phase. After drying at 50 °C for 10 min in an hot air oven, and the spots were detected by spraying with potassium permanganate solution.

Analysis of Sugars in the Crude Extracts of *C. albomarginata* by FTIR

For FTIR analysis, dry ethanolic crude extracts of fresh, dry and rehydrated stages of *C. albomarginata* fronds were used and analysed against the standards of sucrose and trehalose sugars. FTIR spectra were recorded using an IR-spectrometer (Carry 650 FTIR, Agilent Technologies) and mercury cadmium telluride (MCT) detector.

Qualitative Analysis of Sugars by HPLC

HPLC analysis was carried out using a gradient modular pump, Agilent 1200 series injector fitted with a 25 µL loop. For detection, differential refractometer (RI) detector was used and chemstation software (version 6.0) was used. An L-Amine 210 (YMC Co. ltd, Japan) column of 50 x 50 cm was used along with 45 % acetonitrile/water (v/v) with 0.1 M ammonium acetate as the mobile phase at a flow rate of 1.0 mL /min. The run time was 60 min. Qualitative determination was done by comparison of sugars extracted from fresh, dry and rehydrated stages of samples of *C. albomarginata* fronds (as described under TLC section) against the standard mixtures of glucose, sucrose and trehalose sugars.

Extraction of Trehalose from the Fronds of *C. albomarginata* by Hot Ethanol Method

Trehalose was extracted using the modified hot ethanol method of Vázquez-Ortíz, *et al.* [12]. The extracts obtained were re-suspended in 100 µL sterile distilled water and stored at -20 ^{0}C until use.

Trehalase Enzyme Assay: Preparation of crude trehalase enzyme from *Sacchromyces cereviseae.* The wild type strain of *Sacchromyces cereviseae* was used to prepare crude trehalase according to the method given by Rossouw, *et al*. [13] and stored at 4°C until use. The protein content in the crude trehalase preparation was determined by the Bradford method [14] using bovine serum albumin (BSA) as a standard.

Trehalase Assay: The trehalose extracted from *C. albomarginata* (as described above) or the commercial trehalose [2.5 µM (limiting substrate concentration) and 10 µM (saturating substrate concentration)] was incubated with crude trehalase enzyme in assay buffer. The reaction mixture was incubated at 30°C for 1 h and the reaction was stopped by incubating at 90°C for 2 min in a dry bath. The assay mixture was subsequently used for estimating the amount of glucose released by 3,5-Dinitrosalicylic acid (DNS) method [15].

The trehalase assay was performed in two sets: limiting amount of trehalase enzyme (340 µg total protein) and substrate (50 µL of extracted trehalose); saturating amount of enzyme (850 µg) and substrate (100 µL) along with negative controls.

Estimation of Proline during Resurrection of *C. albomarginata* Fronds

The proline content was quantified according to the method of Bates, et al. [16]. The assay was performed with standard proline (50 µmol) and a standard curve was generated. The amount of proline in the test samples was calculated from the standard curve.

Quantitation of Glycine-betaine (GB) during Resurrection of *C. albomarginata* Fronds

The estimation of GB was done according to the method of Greive and Grattan [17]. The assay was also performed with standards of GB (50-200 mg/mL) and the standard curve was generated. The amount of GB in the samples of *C. albomarginata* was determined from the standard curve.

Correlation Analysis of Experimental Data

To study the correlation of resurrection phenomenon with the different experimental factors, the results were analyzed using Microsoft Excel and Statistical Packages for Social Sciences (SPSS 20) statistical analysis software (IBM Corporation, US, version 20) using two replicates of trehalose content, proline content and GB content of *C. albomarginata* resurrection samples. Paired correlation analysis was performed between relative water content (RWC), resurrection time and altitude during different stages of resurrection (fresh, dry and rehydrated stage). Statistical correlative analysis was performed by bivariate correlation using "Pearson correlation coefficients" for two-tailed test of significance with least significant difference (LSD) less than p value of 0.05.

Results

Qualitative Analysis of Sugars Present in Different Stages of Resurrection by FTIR Spectroscopy

C. albomarginata is widely distributed in Himachal Pradesh. In our previous study, we reported the resurrection activity of *C. albomarginata* collected from ten different districts of Himachal Pradesh, India. These locations vary in their climatic conditions and altitude (372 m-2455 m) [10].

Trehalose and sucrose are the major sugars that accumulate during desiccation in plants. Therefore, the FTIR spectra of ethanolic extracts of *C. albomarginata* were compared with those of standard trehalose and sucrose. The position of the major IR peaks in the spectra of fresh, dry and rehydrated stage samples seemed to match with each other (Figure 1 A, B and C). The first predominant IR peak in all the 3 spectra (Figure 1 A-C) was found as a broad peak centered around 3300 cm^{-1}, which corresponds O-H or N-H group. The next five IR peaks were positioned around 2925 cm^{-1} (corresponding to C-H), 2854 cm^{-1} (corresponding to C-H), 1700 cm^{-1} (corresponding to C=O or C-C), 1600 cm^{-1} (corresponding to C=C) and 1020 cm^{-1} (corresponding to C-N or C-X) (Figure 1 A, B and C). Some of the peaks were observed as

small peaks in the IR spectra, indicating the presence of some other interacting functional groups in ethanolic extracts (Figure 1A).

The ethanolic extracts of different stages of resurrection showed no IR peaks at 2995 cm^{-1}, which was only present in the sucrose standard (Figure 1 A-E). Comparison of the FTIR profiles of the resurrection samples with that of trehalose revealed similarities at positions of 3250 cm^{-1}, 2100 $cm,^{-1}$and 1350 cm^{-1} respectively (Figure 1 A-D). These results indicate the possible presence of trehalose, and absence of sucrose in the extracts of fresh, dry and rehydrated stages of fronds of *C.albomarginata*.

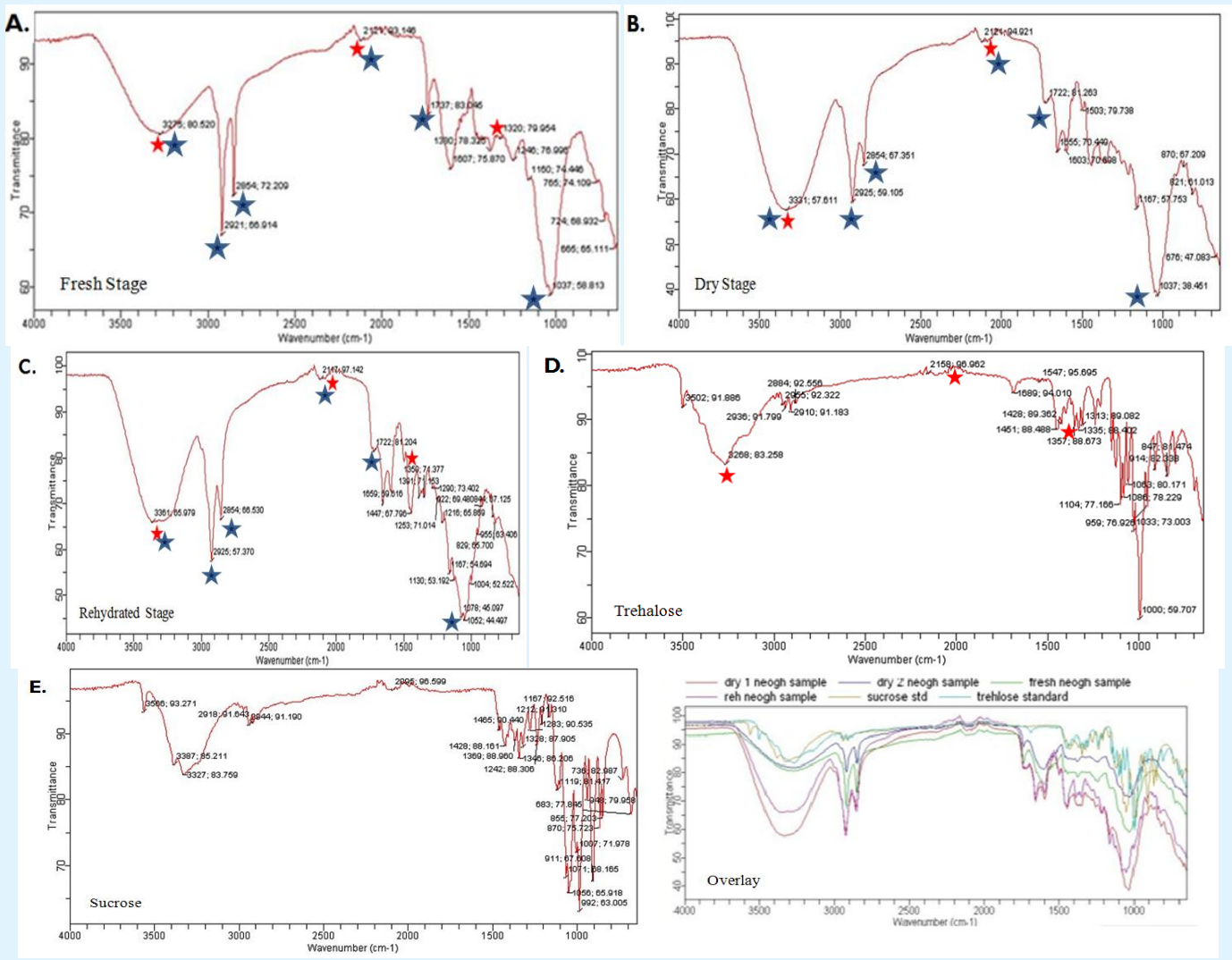


Figure 1: Qualitative analysis of sugars present during different stages (fresh, dry and rehydrated) of resurrection in *C. albomarginata* by FTIR spectroscopy. The crude ethanolic extracts of mature fronds of fresh, dry and rehydrated stages of *C. albomarginata* were subjected to FTIR analysis. Commercial trehalose and sucrose were used as standards. The FTIR spectra are shown for the following samples: **A.** Fresh stage, **B.** Dry stage, **C.** Rehydrated stage, **D.** Trehalose, **E.** Sucrose, and **F.** Overlay of the FTIR spectra of samples shown in **A-E**. Each spectrum is a plot of transmittance value against the wave number range (1000-4000 cm^{-1}). Stars in blue color indicate the common peaks in the resurrection samples, while stars in the red color indicate the common peaks between trehalose and the plant extracts.

Characterization of Sugars in Different Stages of Resurrection in *C. albomarginata* by TLC

To understand the role of sugars in resurrection, trehalose was analyzed in ethanolic extracts during fresh, dry and rehydration stages. In order to detect variation in trehalose content during different stages of resurrection, TLC analysis of the ethanolic extracts of the three stages of resurrection were performed along with trehalose as a standard. TLC analysis was done and developed using $KMnO_4$. As shown in Figure 2, a prominent spot corresponding to the mobility of the trehalose was observed in fresh and dry stages, but not in rehydrated stage of resurrection in *C. albomarginata* ethanolic extracts. Moreover, the intensity of the spot (R_f = 0.84) corresponding to mobility of the trehalose was almost double in the dry stage (R_f = 0.87) as compared to that of fresh stage (R_f = 0.86).

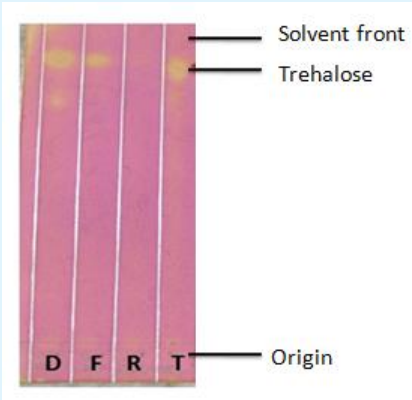


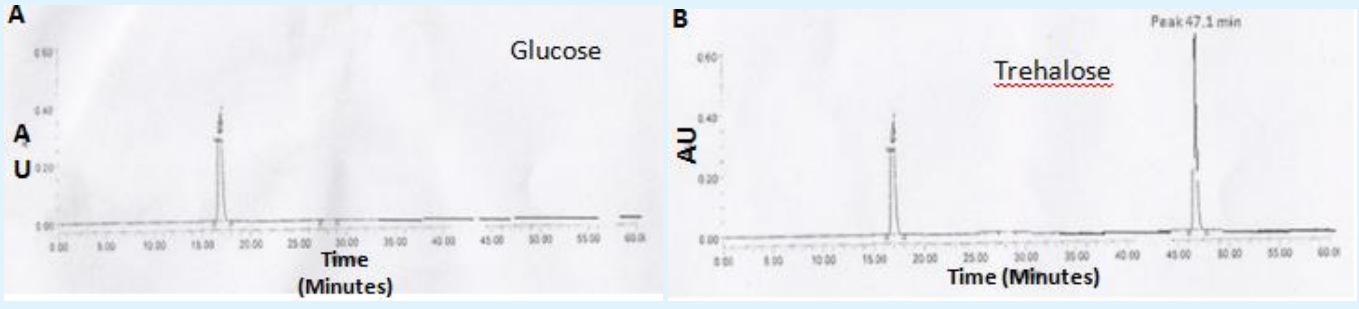
Figure 2: Identification of sugars during different stages of resurrection in *C. albomarginata* by TLC.

These results suggest that trehalose accumulates during dry stage, and gets metabolized during rehydration, resulting in the disappearance of the spot corresponding to trehalose in the rehydrated stage (Figure 2).

Thin layer chromatography analysis of crude ethanolic extracts of *C. albomarginata* samples during fresh (F), dry (D) and rehydrated (R) stages, developed with $KMnO_4$. Yellow spots of sugars were visible in a pink background. The trehalose (T) loaded as reference is indicated.

Qualitative Analysis of Trehalose during Resurrection of C. *albomarginata* by HPLC

The analysis of sugars profile by TLC favor the possibility that trehalose is accumulated by *C. albomarginata* during desiccation/dry condition, and metabolized during rehydration. However, the possible role for other stress sugars like sucrose is still not eliminated. Therefore, a more sensitive analysis was performed by HPLC using trehalose, glucose and sucrose standards. Comparison of the HPLC profiles of ethanolic extracts of C*. albomarginata* fronds from fresh, dry and rehydrated stages with the standards revealed the presence of trehalose in all the three stages. On the other hand, glucose was detected only in fresh and rehydrated stages (Figure 3; Table 1). Several other peaks were present in the ethanolic extracts of C*. albomarginata*, whose identity could not be established (Figure 3 D-F). Quantitative analysis of trehalose content from chromatogram showed that trehalose begins to accumulate during fresh stage (43.6 %); accumulates further at dry stage (46 %); and its level declines during rehydration stage (28 %), possibly due to its utilization as an energy source. Moreover, it was evident from the HPLC chromatograms that sucrose was undetectable during all stages, indicating the role of trehalose as desiccation protectant molecule (Figure 3; Table 1). Consistent with the possible metabolism of trehalose to release glucose, a slight increase in glucose content was observed during the rehydration stage (1.87 %).



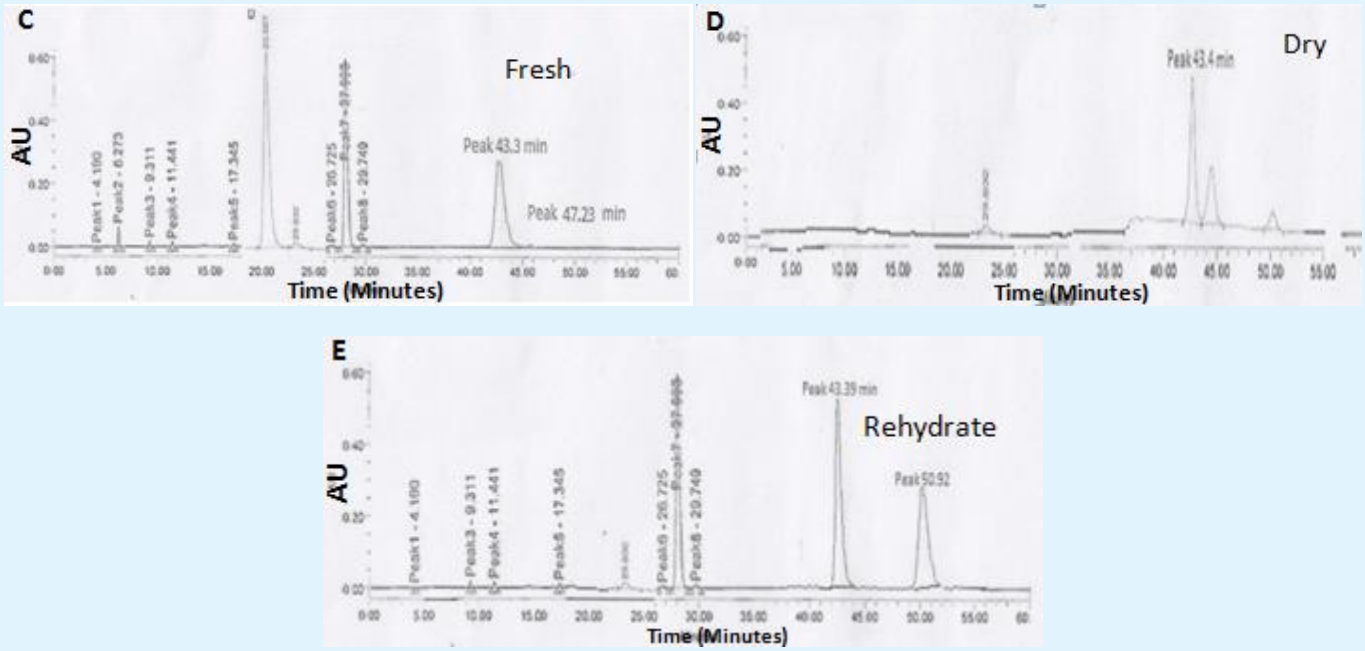


Figure 3: Qualitative analysis of sugars during resurrection stages of *C. albomarginata* by HPLC method. HPLC analysis was performed as described in section 3.10.The chromatograms indicate the detection of sugars in ethanolic extracts of fresh **(C)**, dry **(D)** and rehydrated stage **(E)** fronds of *C. albomarginata*. Chromatograms for the elution of glucose **(A)**, and trehalose **(B)** standards are shown. The absorbance (AU) is plotted against retention time (min).

S. No.	Metabolites	Retention time (min)	(%) area		
			Fresh	Dry	Rehydrate
1	Glucose	17	1.3	-*	1.87
2	Trehalose	43	43.6	46	28

Table 1: List of Sugars, their retention time and percentage area analyzed by HPLC during fresh, dry and rehydrated stages of C. albomarginata fronds.
* - Indicates the absence of sugar in HPLC chromatogram.

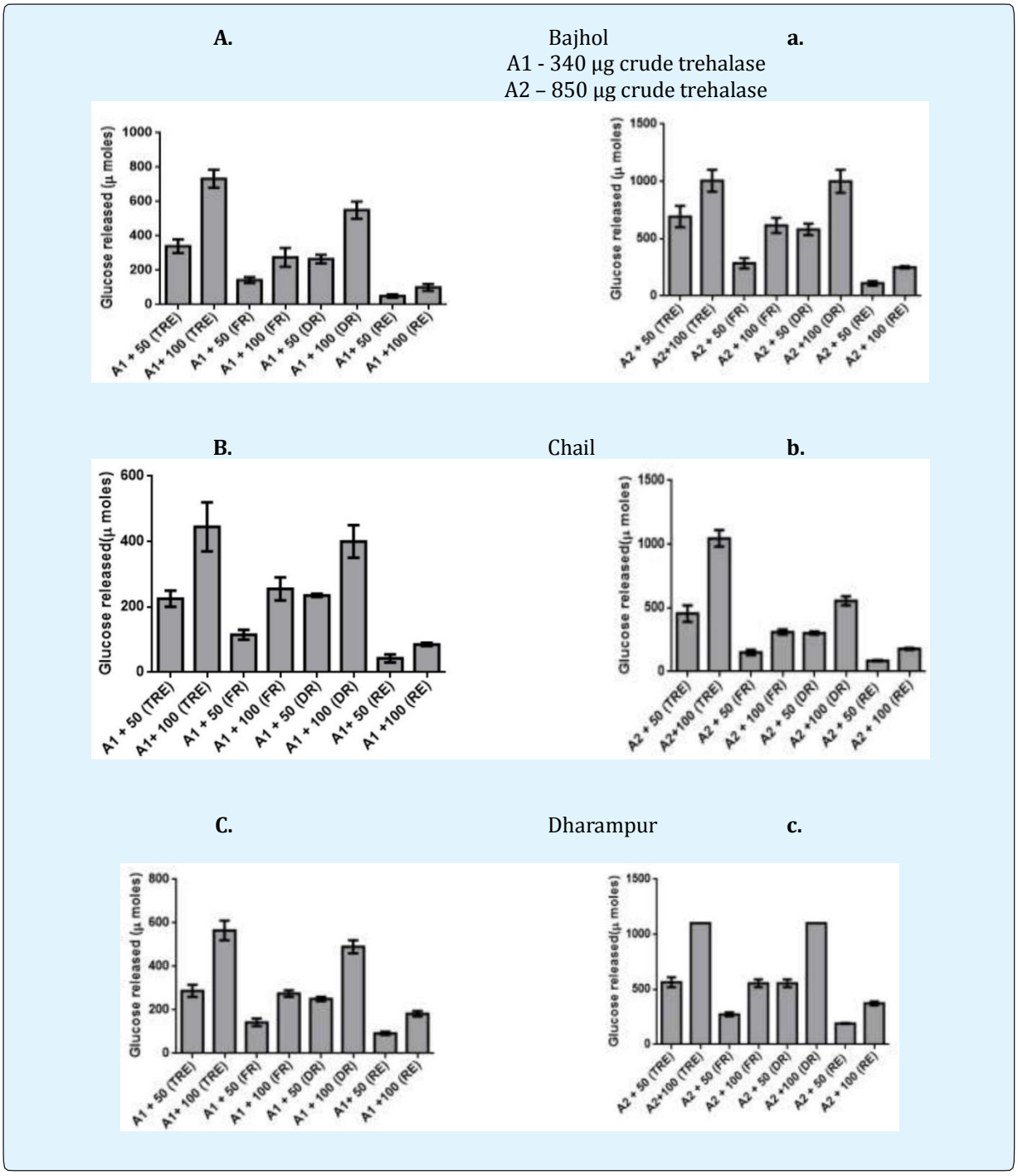
Quantitation of Trehalose Content during different Stages of Resurrection of *C. albomarginata* by Trehalase Assay

The changes in the trehalose content during desiccation and resurrection indicated its role as desiccation protectant in C*. albomarginata.*To quantitate and further validate the amount of trehalose during different stages of resurrection, an enzymatic assay was designed. The conversion of trehalose extracted from different stages of resurrection into glucose was assayed by using a crude preparation of trehalase enzyme from the yeast *Saccharomyces cerevisiae.* The specificity of the assay was confirmed by using appropriate controls (enzyme alone and extracted trehalose alone). The commercial trehalose was used as a positive control for the enzyme assay. The key results from these assays are summarized below:

1) The presence of trehalase in crude enzyme was confirmed by the dose dependent release of glucose upon incubation with purified trehalose (5µM and 10 µM) in all the experiments [A1/A2 + 50/100 (TRE) data in Figure 4 A - J and a – j].
2) The release of glucose was specific to trehalase, since no activity was observed without trehalase.
3) A dose dependent increase in the release of glucose was observed with increasing amounts of enzyme [340 µg and 850 µg; (Figure 4 A-J and a-j)] as well as with increasing amounts of trehalose (50 and 100 µL) extracted from fresh, dry and rehydrated stages of *C. albomarginata* fronds (Figure 4 A-J and a-j).
4) A uniform pattern of trehalose utilization, i.e., amount of glucose released during the three stages of resurrection (fresh, dry and rehydrated)was observed in all the 10 samples (Figure 4 A-J and a-j). It was observed that during rehydration stage, the amount of glucose released was reduced to approximately half as compared to dry stage sample of *C. albomarginata*. Similar results were observed for all the 10 samples of *C. albomarginata* (Figure 4 A-J and a-j). Together, these results confirm the role of trehalose in the resurrection activity of *C. albomarginata*

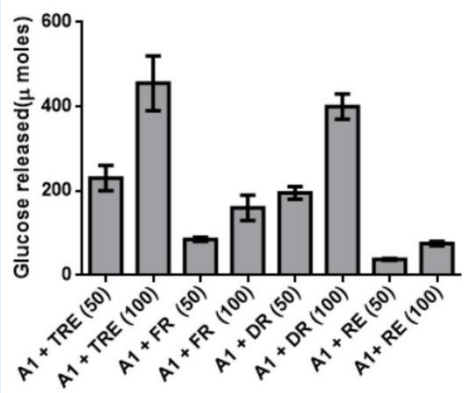
and its utilization during rehydration.

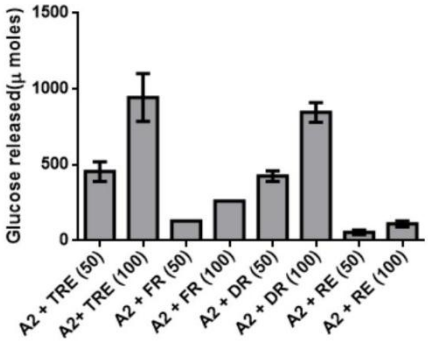
5) The amount of glucose released from trehalosein dry stage of all the 10 samples of *C. albomarginata* was almost double the amount when compared to fresh stage of respective sample (Figure 4). These results indicate the accumulation of trehalose during dry stage, a feature observed in many resurrection plants during desiccation. Thus, qualitative (TLC, FTIR and HPLC) and quantitative (trehalase assays) experiments strongly indicate a role for trehalose in resurrection of *C. albomarginata* fronds.



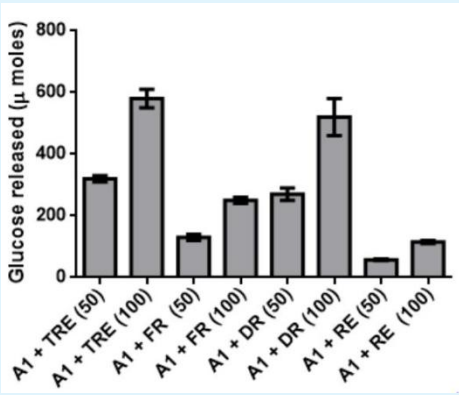
D. Kasauli **d.**

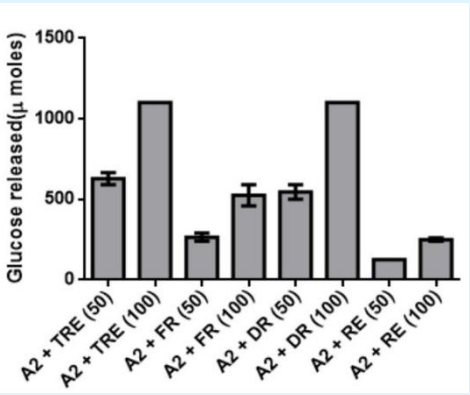
A1- 340 µg crude trehalase
A2– 850 µg crude trehalase



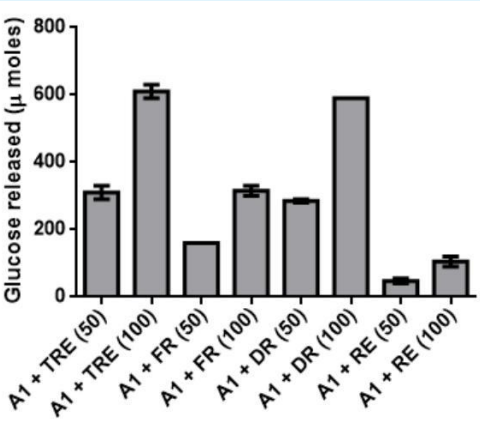


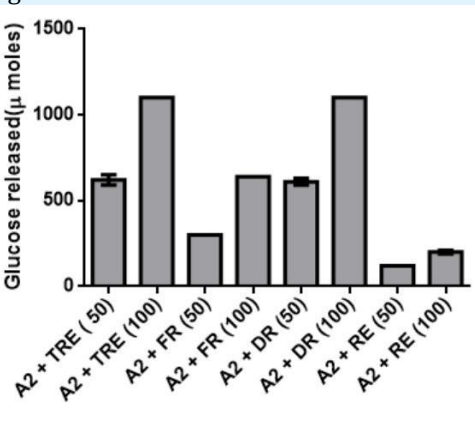
E. Mandi **e.**





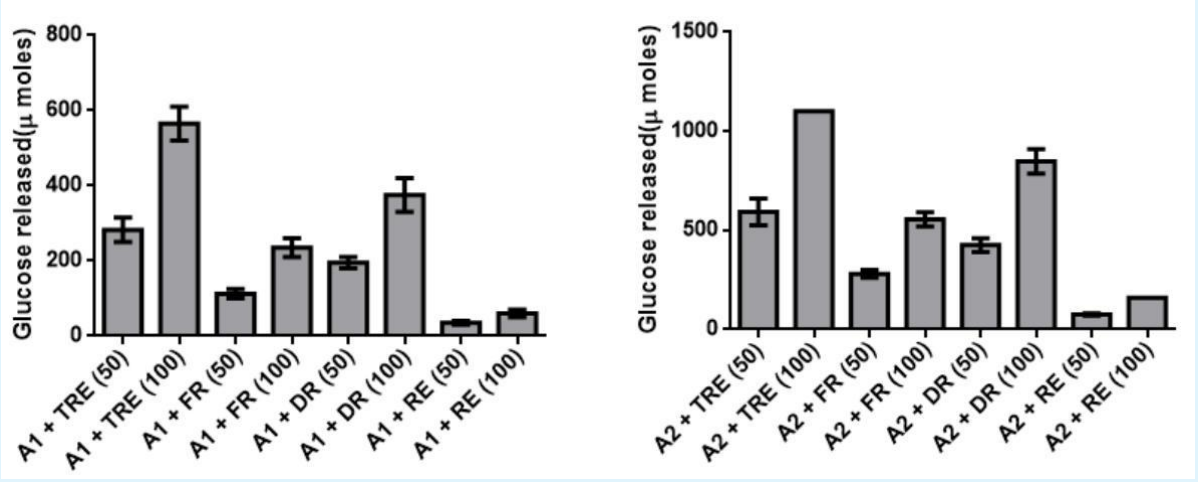
F. Nalagarh **f.**



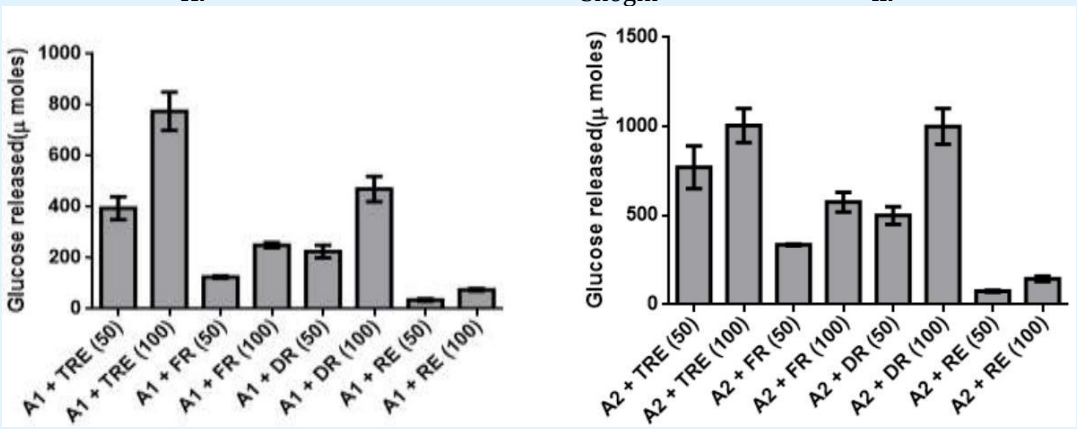


G. Shimla **g.**

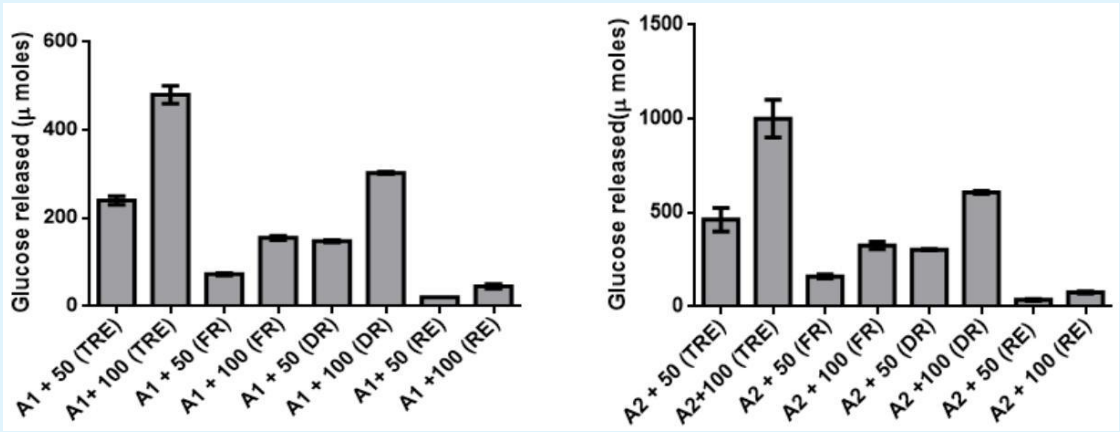
A1- 340 µg crude trehalase
A2- 850 µg crude trehalase



H. Shoghi **h.**



I. Sirmour **i.**



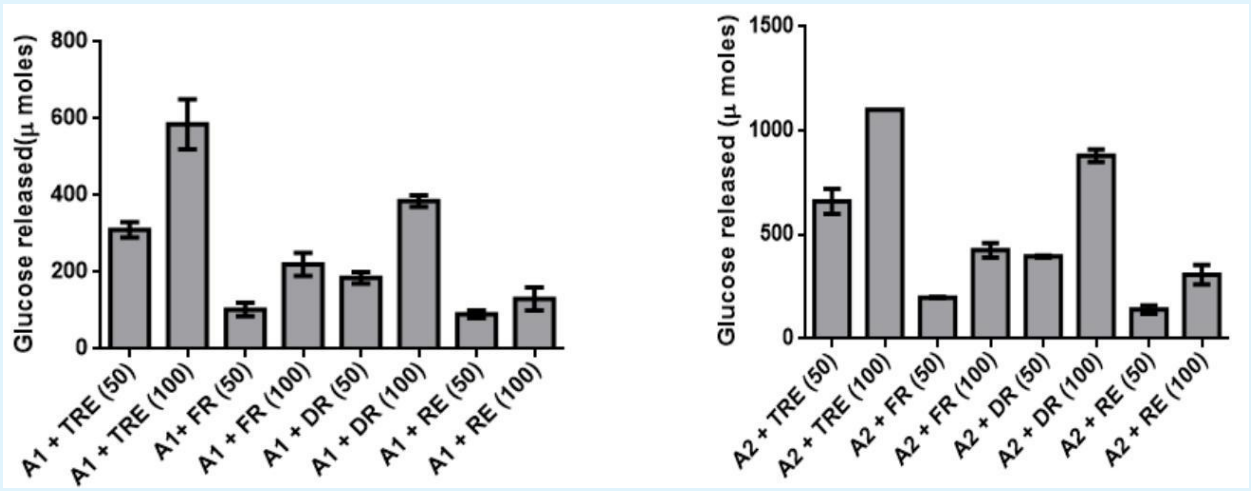


Figure 4: Estimation and comparison of trehalose content during resurrection of *C. albomarginata* collected from Solan and adjacent areas of Himachal Pradesh. *C. albomarginata* fronds were collected from Bajhol (**A and a**), Chail (**B and b**), Dharampur (**C and c**), Kasauli (**D and d**), Mandi (**E and e**), Nalagarh (**F and f**), Shimla (**G and g**), Shoghi (**H and h**), Sirmour (**I and i**), Subathu (**J and j**). Trehalase assay was done with two different amounts of enzyme, i.e., 340 µg crude enzyme (**A1**) (**panel A-J**) and 850 µg (**A2**) (panel **a-j**) with all the ten samples. For every sample of *C. albomarginata*, assays were done with purified trehalose (5 µM and 10 µM), which served as positive control (indicated as A1/A2 + TRE (50) or A1/A2 + TRE (100), respectively). For the resurrection samples, trehalase assay was done with 50 µL and 100 µL of trehalose extracted from fresh [A1/A2+ FR (50/100)], dry [A1/A2+ DR (50/100)] and rehydrated [A1/A2+ RE (50/100)] stages. The amount of glucose released (µ moles) was plotted against the indicated reaction set for each location. Error bars denote SD for 3 independent experiments.

Quantitative Analysis of Proline Content during Resurrection of *C. albomarginata*

The amino acid proline has been found to accumulate in the tissues of several plants during desiccation [18]. To study the role of proline in the resurrection of *C. albomarginata,* proline content was measured in all the 10 samples during different stages of resurrection as described above. Proline was detected in the fresh stage of *C. albomarginata* fronds of all the 10 locations, ranging from 1.1 µmol/g in Shimla to 1.85 µmol/g in Bajhol sample (Figure 5A-J). An increase in proline content (59 – 137 %) was observed in the dry stage, respectively of *C. albomarginata* fronds of all the ten locations relative to the fresh stage (Figure 5A-J; fresh versus dry). These results indicate that proline accumulates in response to desiccation stress. Interestingly, amount of proline in rehydrated stages of all the samples locations of *C. albomarginata* was only marginally reduced (62-156 %) relative to the proline content in their respective dry stages (Figure 5A-J; dry versus rehydrated). These results suggest that proline accumulates during dry stage and its levels are maintained during rehydration, indicating its role as an osmoprotectant during rehydration and desiccation. The pattern of proline accumulation during different stages of resurrection of *C. albomarginata* collected from ten different locations was consistent with each other (Figure 5A-J).

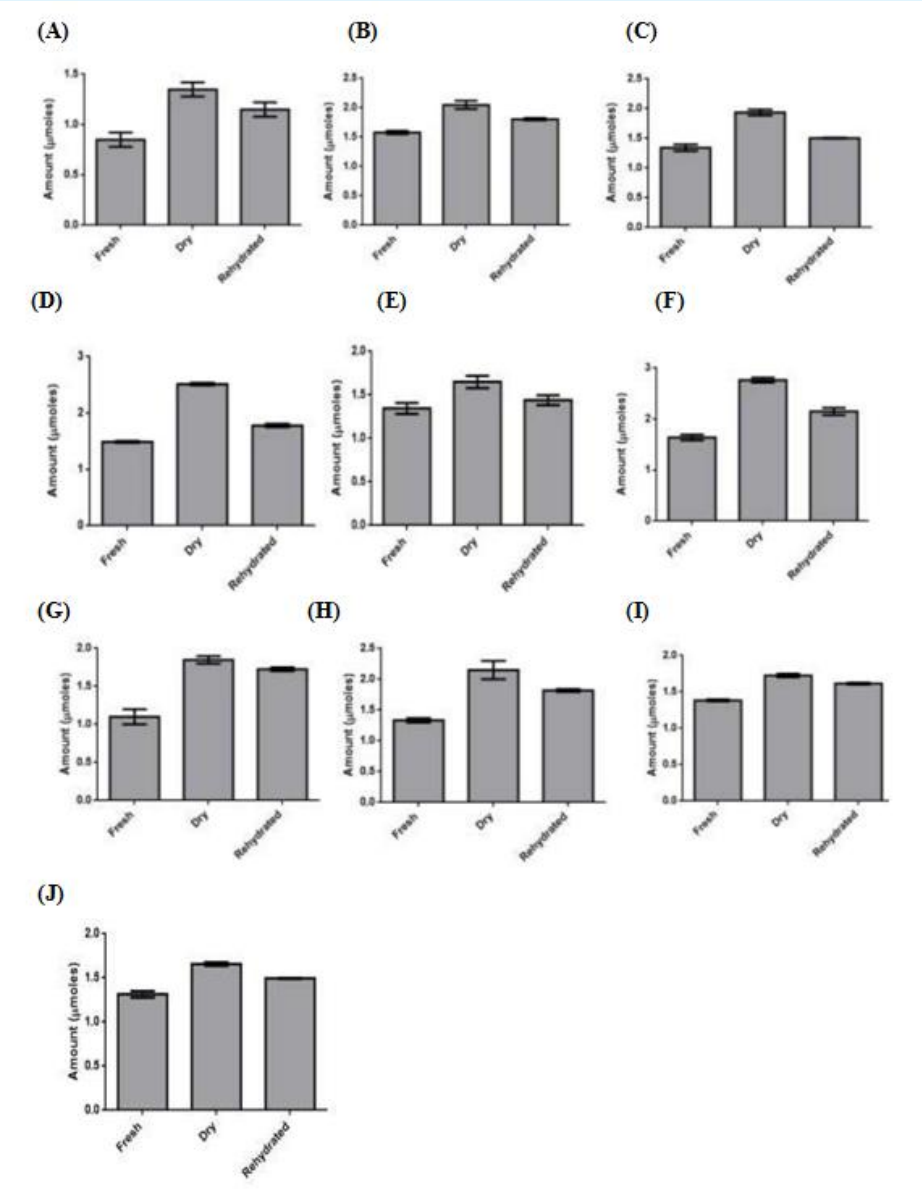


Figure 5: Estimation of proline content during different stages of resurrection in *C. albomarginata*. Proline content was measured in the 3 stages of resurrection (fresh, dry and rehydrated) for each sample. The amount of proline (µmoles) was plotted against the indicated stages of the samples: (A) Bajhol, (B) Chail, (C) Dharampur (D) Kasauli, (E) Mandi, (F) Nalagarh, (G) Shimla, (H) Shoghi, (I) Subathu, (J) Sirmour. Error bars denote SD for 3 independent experiments.

Quantitative analysis of Glycine Betaine (GB) Content during Resurrection of *C. albomarginata*

Glycine betaine is known to accumulate as osmolyte in several plant species during desiccation [19]. Extreme dryness or desiccation is one form of stress that can induce GB production and accumulation, and in some cases, accumulation is considered to be an adaptive response [19]. GB accumulation was analysed during resurrection of *C. albomarginata.* The amount of GB in different stages of resurrection of *C. albomarginata*

samples collected from 10 different locations was determined.

Glycine betaine could be detected in all the three stages of resurrection in *C. albomarginata* collected from the ten locations (Figure 6, A-J). The amount of GB during the fresh stage was lowest (14 µg) in the Bajhol sample, while highest in (25 µg) in Sirmour sample (Figure 6). Upon drying, a significant increase in the levels of GB was observed for all the ten samples, which ranges from 121 to 192 % relative to the levels in the fresh stage (Figure 6). These results indicate that GB accumulates in response to dryness in the fronds of *C. albomarginata.* Similar to the proline, most of the GB (80 %) content present in the dry stage was retained during rehydration stage of *C. albomarginata* collected from the ten different locations (Figure 6). These results suggest that GB accumulates during dryness/desiccation and retained during rehydration of *C. albomarginata* fronds. Together both proline and GB appear to serve as osmoprotectant during resurrection of *C. albomarginata.*

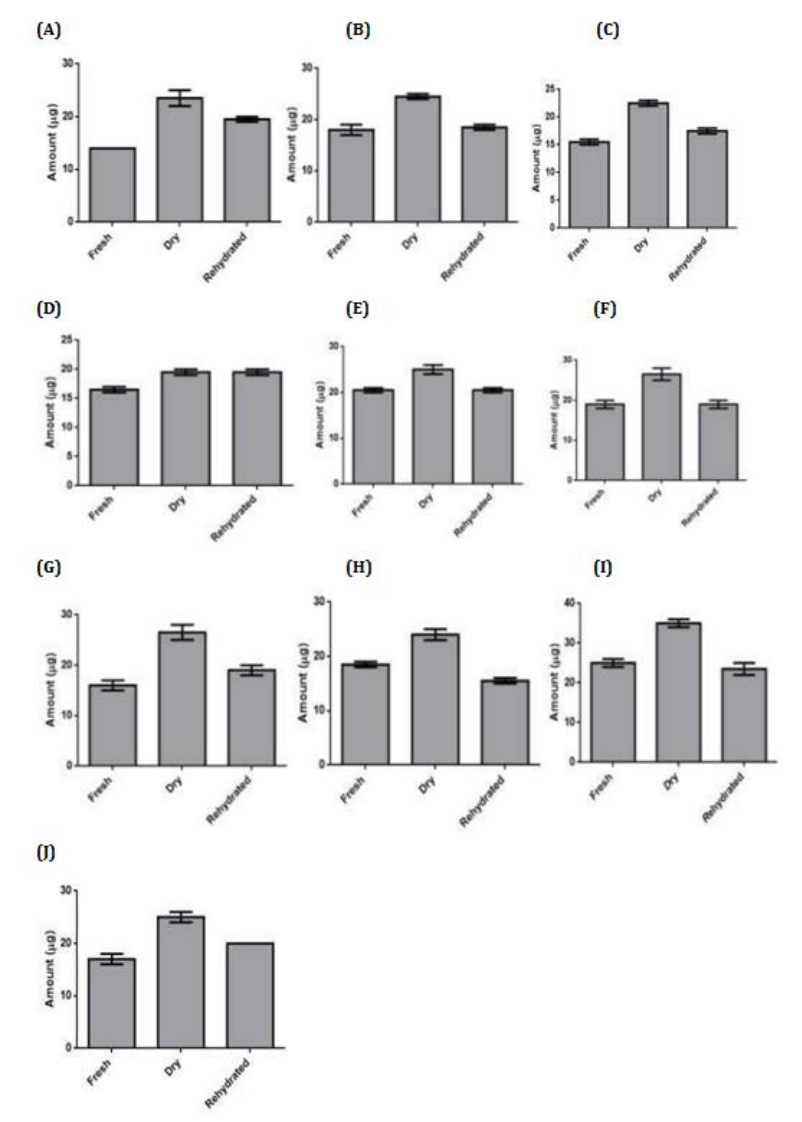


Figure 6: Quantification and comparison of Glycine betaine content during resurrection of C. albomarginata. GB content was measured in the three stages of resurrection (fresh, dry and rehydrated) for each sample. The amount of GB (µg) was plotted against the indicated samples: (A) Bajhol, (B) Chail, (C) Dharampur (D) Kasauli, (E) Mandi, (F) Nalagarh, (G) Shimla, (H) Shoghi, (I) Subathu, (J) Sirmour. Error bars denote SD for 3 independent experiments.

Correlation Analysis of Physical and Biological Factors with Resurrection of *C. albomarginata*

To study the relationship between resurrection phenomenon and the physiological parameter involved, paired correlation analysis was performed between accumulation of metabolites such as trehalose, proline and glycine betaine, and physiological factors such as relative water content, time taken for resurrection, temperature and altitude. Three types of correlations were observed: positive, negative or no correlation (Table 2). The trehalose content in fresh stage of *C. albomarginata* had no correlation with either of the physiological parameters tested (Table 2). On the other hand, trehalose content in dry stage showed inverse correlation with RWC, and the altitude of the location. Similarly, during rehydration condition, trehalose content negatively correlated with RWC (the capacity to obtain full turgidity).

S. No.	Parameters	RWC	Resurrection time	Temp	Altitude
A.	**Trehalose content**				
1	Fresh stage	NS*	NS	NS	NS
2	Dry stage	- S*	NS	NS	- S
3	Rehydrated stage	- S	NS	NS	NS
B.	**Proline content**				
1	Fresh stage	NS	NS	NS	NS
2	Dry stage	+ S*	NS	NS	NS
3	Rehydrated stage	NS	- S	NS	NS
C.	**Glycine-Betaine content**				
1	Fresh stage	NS	NS	NS	NS
2	Dry stage	NS	NS	NS	NS
3	Rehydrated stage	NS	NS	NS	NS

Table 2: Correlation analysis between various physiological parameters and metabolite accumulation during different stages of resurrection of *C. albomarginata*.
*(NS): Not significant, i.e., no significant correlation.
*(+S): Positive Significant value, i.e., positive correlation.
*(-S): Negative Significant value, i.e., negative correlation.

The correlation analysis of stress amino acid proline did not indicate any correlation with any of the parameter during fresh stage, whereas during dry stage, accumulation of proline showed a direct correlation with RWC (Table 3). During rehydration stage, proline content showed a negative correlation with resurrection time (Table 3). GB content did not show any correlation during fresh stage, dry and rehydrated stages with any of the parameters studied (Table 3).

S.No.	Metabolite	Stage of resurrection	Qualitative analysis*	
			Presence/ Absence	Accumulation /utilization
1	Trehalose	Fresh	(+)	(+++)
		Dry	(+)	(+++)
		Rehydrated	(+)	(---)
2	Proline	Fresh	(+)	(+++)
		Dry	(+)	(+++)
		Rehydrated	(+)	(+++)
3	Glycine Betaine	Fresh	(+)	(+++)
		Dry	(+)	(+++)
		Rehydrated	(+)	(+++)

Table 3: Qualitative analysis of metabolites during resurrection activity in *C. albomarginata*.
* (+), Presence; (-), Absence; (+++), Accumulation; (---), Utilization

Discussion

Resurrection is the ability of an organism to survive the loss of most ~ 95% of its cellular water for extended periods and to recover full metabolic competence upon rehydration. The plant kingdom has many unique groups of plant species, ranging from lower to higher groups. Among others, only a few can tolerate extreme water loss (desiccation) of down to 5% of cellular water content for longer periods without dying, commonly referred to as resurrection plants [5,20-24].

Desiccation tolerance (DT) in resurrection plants is a multi-genic and multi-factorial phenomenon and is associated with prevention of damage caused by oxidation. Resurrection plants produce a high diversity of metabolites for defense against desiccation and are required for growth and developmental processes during lethal conditions e.g. osmoprotectants, such as sugars (trehalose, sucrose and fructan), amino acids (tryptophan and proline) and ammonium compounds (polyamines and glycinebetaine) [25]. These molecules accumulate in these plants under lethal conditions arise due desiccation as adaptive mechanism and provide stress tolerance. Thus, metabolites play a key role during desiccation stress and revival upon rehydration [9].

Thus, to characterize the metabolites essential for resurrection in *C. albomarginata*, quantitative and qualitative analysis of various metabolites such as trehalose, proline and glycine betaine during dehydration/desiccation and rehydration were performed.

In this study, techniques like TLC, FTIR and HPLC were used as the major techniques for the characterization of sugars, while enzymatic and colorimetric assays were used for the quantitative estimation of trehalose, proline and glycine betaine during resurrection.

In TLC analysis, the appearance and disappearance of spot corresponding to trehalose was observed in the crude dehydrated and rehydrated samples, respectively. The disappearance of trehalose during rehydration might indicate its utilization by the plant as an energy source during rehydration process. Quantitative estimation of extracted sugars by HPLC analysis from fresh, dehydrated and rehydrated stages of *C. albomarginata* showed that trehalose levels were highest in dehydrated stage (46 %), which reduced to 28% upon rehydration. Thus, HPLC data further support the TLC results. FTIR analysis of intracellular sugars in crude extracts of *C. albomarginata* during fresh, dehydrated and rehydration stages indicated the closest match of the sugar in these samples with trehalose standard rather than sucrose standard. Quantitative estimation of extracted trehalose sugar from the fronds of fresh, dry and rehydrated stages of *C. albomarginata* was done using trehalase assay. Interestingly, the pattern of trehalose accumulation was consistent with results obtained from TLC and HPLC analysis, in all *C. albomarginata* samples collected from ten different locations (Fig 4). Thus, trehalose appears to be the major sugar accumulating during dehydrated condition, and might have a protective role in *C. albomarginata* during desiccation.

Proline plays multiple roles as stress protectant, viz, stabilizing enzymes, membranes, proteins and free radical scavengers. Besides acting as an excellent osmolyte, it also acts as a metal chelator, an antioxidative defense molecule and a signaling molecule. Proline accumulation is a common phenomenon observed in response to environmental stress in many organisms including plants [26]. In plants, intracellular proline levels have been found to increase by > 100-fold during stress [27]. Proline accumulation in plants occurs during exposure to various stresses, including salt [28], drought [29], UV radiation [30], heavy metal ions [31], pathogens [32], and oxidative stress [33].

The level of proline accumulation during dehydration stage varied significantly amongst the 10 samples of *C. albomarginata* (Figure 5). The highest proline accumulation was observed in Nalagarh sample (2.7 µmol), followed by Kasauli sample (2.5 µmol) and Shoghi sample (2.1 µmol), respectively. The lowest proline accumulation was observed in Bajhol sample (1.35 µmol).

GB protects cells from stresses by maintaining an osmotic balance with the surrounding environment [34] and by stabilizing the quaternary structures of complex proteins. In most plants, Glycine betaine acts as an osmolyte and provides protection to the plant by scavenging of reactive oxygen species (ROS) to restore redox metabolism, and mediates preservation of cellular turgor by restitution of osmotic balance.

In this study, GB content was estimated in all the collected samples of *C. albomarginata* during fresh, dehydrated and rehydrated stages of resurrection, to understand the role of GB. Similar to proline, GB accumulated during dehydration stage in all the 10 samples of *C. albomarginata*, and remained largely unaltered during rehydrated state, indicating a protective role for GB during resurrection (Figure 6). The level of GB accumulated during dry stage varied significantly amongst the 10 samples of *C. albomarginata*; the highest GB accumulation was observed in Sirmour sample (35

µg), followed by Nalagarh sample (26.5 µg) and Shimla sample (26.5 µg), respectively. The lowest GB accumulation was observed in Kasauli sample (19.5 µg).

Correlative analysis between trehalose accumulation and the RWC indicated that in fresh stage, there was no significant correlation of the trehalose content with RWC content and resurrection time, while in dehydrated stage, the accumulation of trehalose showed inverse relation with RWC. In addition, accumulation of trehalose sugar during dehydrated stage had a significant inverse correlation with altitude of the place of collection of *C. albomarginata* fronds. No such reports are available in literature for comparison. The correlation analysis of proline content did not show any correlation with any of the factors during fresh stage, whereas during dehydrated stage, accumulation of proline showed a positive correlation with RWC, i.e., lesser the water content in the cell or higher the RWC, more is the synthesis and accumulation of enzyme/membrane/protein stabilizers like proline during dehydrated conditions. During rehydration stage of resurrection of *C. albomarginata*, proline content showed an inverse correlation with resurrection time; hence more the proline content during the rehydrated stage, faster is the resurrection of the plant. The accumulation of GB did not show any correlation with RWC, resurrection time and altitude in either of the three stages of resurrection. The GB has generalized protective role again oxidative stress in all the plants. Further studies are required to confirm its role during resurrection.

Conclusion

From the present study, it can be concluded that various metabolites accumulated during dehydrated stage of *C. albomarginata* in response to physiological conditions and protect the plant from the detrimental effects of the desiccation. In addition, during rehydration, few metabolites were used up (example, trehalose) for resurrection activity, whereas others were maintained as osmoprotectants (proline and GB). This study has revealed that the fern *C. albomarginata* is sensitive to dehydration stress, and relies on readily inducible protection to combat desiccation. A detailed characterization in future of the genomics and proteomics of resurrection phenomenon should help in better understanding of the adaptive mechanisms developed by resurrection plants and identify the role of metabolites involved in stress tolerance.

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