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ARTICLE

Effects of Desiccation on Germination and Biochemistry in Seeds of *Hydnocarpus alpina* Wight, an Endemic Medicinal Tree Species of Southern Western Ghats, India

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Abstract

Seeds of *Hydnocarpus alpina* Wight are desiccation sensitive. Fresh seeds on harvest showed 41.3% moisture content with 63% germination. However, after desiccation for 144 hours at open laboratory conditions (28±2°C and 65% RH) their moisture content and percentage of germination decreased to 9.4% and 8.3% respectively. Associated with the desiccation of the seeds, following biochemical and physiological changes were observed. The peroxidase and polyphenol oxidase enzymes, which showed an enhanced activity during the initial period of drying, later decreased as the moisture content level dipped to 30%. The sugar and phenol contents increased during desiccation, while lipids and starch content decreased. A decrease in the protein content noticed during the initial period of drying, flattened out later, and did not record any difference on prolonged desiccation. Also, the seed leachate carried an increased concentration of solutes. Further, no significant correlation was noticed in amino acid and protein levels with desiccation. These results emphasized that, sequential loss in moisture content leads to drastic change in biochemistry, resulting in the loss of seed viability.

Keywords: Hydnocarpus, Desiccation, Germination, Biochemistry, Peroxidase, Poly Phenol Oxidase

Introduction

Hydnocarpus alpina Wight (Family-Flacourtiaceae) is a tropical dioecious avenue tree species attaining a height of 20-30m and a girth of 0.6 - 1.2m, occurring as endemic in the forests of Southern Western Ghats of peninsular India from South Kanara to Kerala. Flowers are pale yellow in racemes. Flowering from November to December and fruiting from March to May. The seeds of several species of Hydnocarpus, as well as some other genera of Flacourtiaceae, yield fatty oils, generally known as Chaulmoogra oil, used extensively in the treatment of leprosy and other cutaneous diseases (Anonymous,1959).

Seeds of many tropical and subtropical tree species are characterized with high moisture content and are intolerant of desiccation (Roberts and Ellis, 1982). They have been termed recalcitrant by Roberts (1973). The seeds of *H. alpina* belong to this group. However, substantial progress has been made in our understanding of the biochemistry of recalcitrant seeds and the various problems associated with their longevity. The loss of viability in recalcitrant seeds is the synergistic effect of a wide range of metabolic processes. These include mechanical stresses induced by the removal of water which can cause structural changes at sub cellular, cellular and at tissue levels. King and Roberts (1979) suggested that the activities of metabolites in the recalcitrant seeds are altered by drying, resulting in the loss of biological activity. Thus the quantification of these biomolecules would serve as the markers in ascertaining the seed viability. Therefore an attempt is being made to evaluate the changes in moisture content, germination, leachate conductivity, biochemistry including antioxidant enzymes during desiccation of *Hydnocarpus alpina* seeds, which in turn can be used as metabolic markers for detecting desiccation sensitive seed viability loss.

Materials and Methods

Fruits of *Hydnocarpus alpina* Wight were collected, at a stage when the fruit wall showed striped condition on maturity, from forest patches of Kallar-Ponmudi area of Thiruvananthapuram districts (Southern region of Western Ghats) in three consecutive years from 1998 to 2001. The pulpy seeds were cleaned with tap water, followed by distilled water and then surface dried in an open laboratory condition at 28±2^{0}C and 65% RH. Moisture content of the whole seed (including pericarp) was determined by monitoring weight loss during drying and by the High Constant Temperature Oven Method (ISTA, 1985). Ten seeds in triplicate were cut and weighed before and after drying at 130±2^{0}C for 1 hour and the moisture content was calculated as the

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percentage of water on fresh weight basis. The viability of seeds was determined on the basis of the percentage of germinated seeds. The seed was scored as germinated when the pericarp opens and the radicle comes out to a length of 5mm (ISTA, 1993). Germination test was carried out in three replicates of 100 seeds each, rolled in acid free germination paper kept in seed germinator conditions 30±2^{0}C and 65% RH. For desiccation treatments, seeds were kept at open laboratory conditions (28±2^{0}C and 65% RH) and taken for different analysis in a regular period of time (0 to 144 hours). Electrolyte leakage was tested by taking three replicates of ten seeds for each lot in a glass beaker covered after adding distilled water (25.0ml each) to reduce evaporation and contamination by dust and were kept in normal laboratory condition (28±2^{0}C and 65% RH). After 24 hours the seeds were removed, the electrical conductivity of seed leachate was measured using a dip cell conductivity meter (Systronics, DDR, type 306). Specific conductance is expressed as mscm^{-1}g^{-1}.

Biochemical extraction and estimation

One gram fresh tissue sample from different desiccation periods was homogenized in 80% ethanol. Homogenate was then centrifuged at 3000rpm for 10 minutes. The residue was washed again with 80% ethanol. The volume of pooled supernatant was noted and served as the source for estimation of phenol (Swain and Hillis, 1959) and amino acid (Sadasivam and Manickam, 1996). A known volume of combined ethanol fraction was evaporated to dryness and then the residue was re-dissolved in known volume of distilled water by using a fine polished glass rod and served as the source for total soluble sugar estimation (Montgomery, 1957). The left over residue was ground with 30 and 15% Per Chloric Acid (PCA) respectively at two times, centrifuged at 3000rpm for 10 minutes each and combined supernatant used for the estimation of starch (Mc Cready *et al.*, 1950). For protein estimation, the tissue was homogenized in double distilled water and precipitated with Tri Chloro Acetic acid (TCA) and estimated following Lowry *et al.*, (1951).

Lipid extraction and estimation

Lipids were extracted following Bligh and Dyer (1959). One gm sample was homogenized in a mixture of chloroform and methanol (2:1v/v) and kept over night at room temperature in dark. Further addition of 20.0mL chloroform and 20.0mL distilled water was made and centrifuged at 5000rpm for 15 minutes. Of the three layers, the clear lower layer of chloroform containing all lipids was carefully collected evaporated and the amount of lipid was determined gravimetrically.

Enzyme extraction

Seeds without the testa (1.0gm) at different desiccation periods were homogenized in a pre chilled mortar and pestle with a pinch of purified sand and 5.0mL of chilled phosphate buffer (0.1 M and pH 7.0). The homogenate was centrifuged at 3000rpm for 10 minutes. The residue was re-suspended again in 5.0mL of homogenizing buffer, centrifuged and the combined supernatant served as the source of enzymes peroxidase and polyphenol oxidase. All the enzymatic operations were carried out at 4°C.

Peroxidase assay (H2O2 Oxidoreductase EC.1.11.1.7)

Peroxidase activity was measured according to Chance and Maehly (1955) recording the change in absorbency at 470nm due to oxidation of guiacol in the presence of hydrogen peroxide. The assay mixture consisted of 1.0mL guaiacol (0.02M), 3.0mL phosphate buffer (0.1M, pH.7.0) and 0.5mL of the enzyme. The reaction was started by addition of 0.5mL hydrogen peroxide (13mM) and change in absorbance was recorded in a Spectrophotometer (Systronics model 106) at 470nm and expressed as change in absorbance min-1 mg protein-1.

Polyphenol oxidase assay (Mono phenol, dihydroxy phenylalanine: Oxygen Oxidoreductase EC.1.14.18.1)

The method of Yamaguchi *et al.*, (1970) was followed to estimate polyphenol oxidase (O-diphenol oxidase) activity. The assay mixture consisted of 0.01M catechol and 0.1M proline in 0.1M phosphate buffer (pH 6.8). Enzyme extract (0.5mL) was added, mixed quickly and change in absorbance at 525nm was observed in a Systronics model 106, Spectrophotometer. The enzyme activity was calculated as the initial linear change in absorbance min^{-1} mg protein^{-1}.

Statistical analysis

Linear regression analysis was done by taking independent variable and dependent variable as, desiccation vs moisture content, desiccation vs germination, desiccation vs conductivity, germination vs moisture content, conductivity vs moisture content and conductivity vs germination, separately following the equation Y= a+bx. Correlation coefficient (r) have been calculated and there significance as per Sokal and Rohlf (1981).

Results

Moisture content, germination and conductivity

Fresh seeds when collected had 41.3% moisture (fresh weight basis) and 63% germination. Under open laboratory conditions, seed moisture content and germination percentage decreased considerably. After 12 hours the seed moisture content became 35.1% with only 40% germination. After 24 hours, moisture content decreased to 30% with a corresponding germination of 26%. After 144 hours of desiccation the percentage of germination was only 8.3 with 9.4% moisture content (Fig 1). Germination was not recorded below 9% moisture content. With the decrease in moisture content during desiccation, the seed leachate conductivity increased gradually from 0.267ms cm^{-1}g^{-1} at 0 time to 0.650ms cm^{-1}g^{-1} after 144 hours of desiccation (Fig 1).

Figure 1 (Y = a+bx)

a) Correlation between Desiccation (hrs.) and Moisture content (%) of *H.alpina* seeds; r=0.95, p<0.001

b) Correlation between Desiccation (hrs.) and Germination (%) of *H. alpina* seeds; r=0.83, p<0.01

c) Correlation between Desiccation (hrs.) and conductivity (ms/cm/gm) of *H. alpina* seeds; r=0.96, p<0.001

d) Correlation between Germination (%) and Moisture content (%) of *H. alpina* seeds; r=0.92, p<0.001

e) Correlation between conductivity (ms/cm/gm) and Moisture content (%) of *H. alpina* seeds; r=0.95, p<0.001

f) Correlation between conductivity (ms/cm/gm) and Germination (%) of *H. alpina* seeds; r=0.86, p<0.001

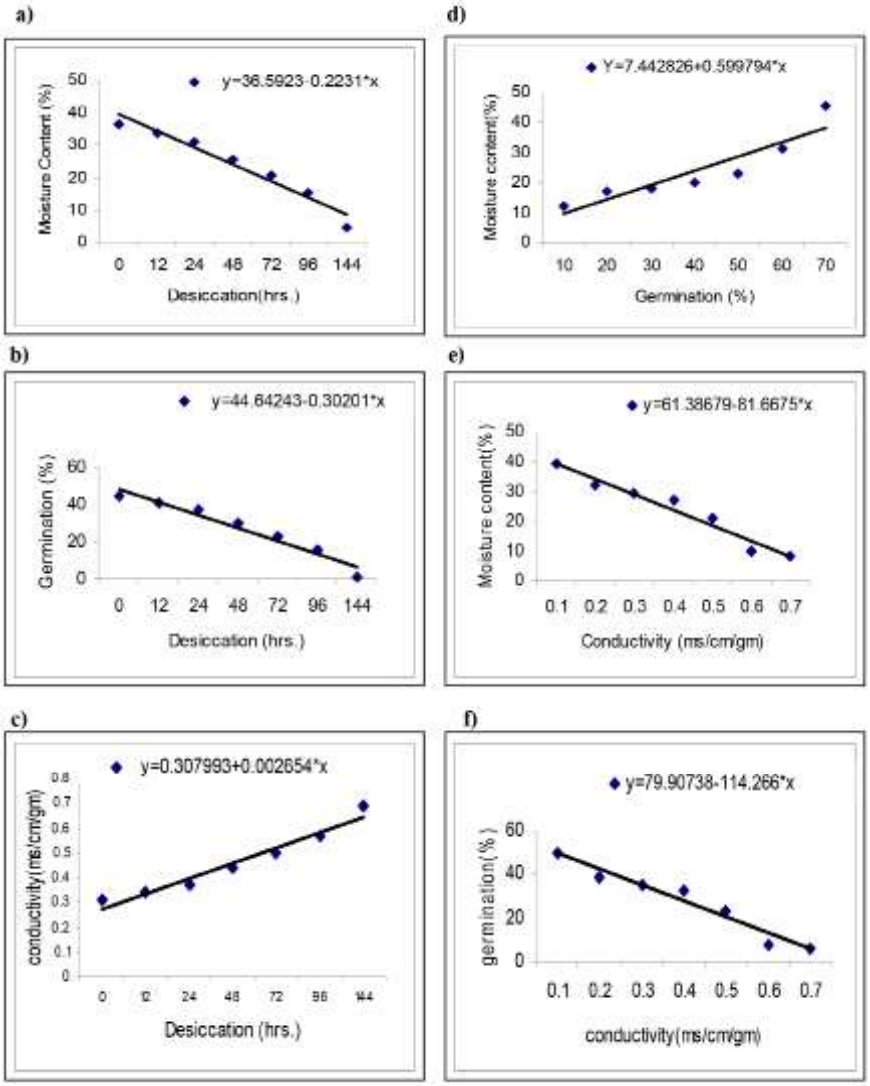

Table 1. Changes of primary metabolites in seeds of *Hydnocarpus alpina* during desiccation

Desiccation period (hours)	Total Soluble Sugars (TSS) (mg/gdw)	Starch (mg/gdw)	Protein (mg/gdw)	Amino acid (mg/gdw)	Lipid (mg/gdw)	Total Phenols (mg/gdw)
0	2.971 ±05	5.36 ±0.08	37.6 ±0.5	5.3 ±0.10	305 ±0.125	4.75 ±0.05
12	5.18 ±0.15	5.32 ±0.03	21.9 ±1.06	8.1 ±0.20	308 ±1.30	8.64 ±0.80
24	8.32 ±0.30	5.25 ±0.09	22.3 ±0.33	8.6 ±0.20	303 ±0.109	9.13 ±0.12
48	11.2 ±0.26	5.04 ±0.60	22.9 ±0.10	8.63 ±0.15	301 ±1.00	9.16 ±0.15
72	14.32 ±0.22	4.93 ±0.04	23.1 ±0.50	8.7 ±0.10	300 ±0.403	10.1 ±0.60
96	16.58 ±0.50	4.36 ±0.07	24 ±0.40	6.9 ±0.10	292 ±0.224	10.4 ±0.30
144	20.42 ±0.30	4.25 ±0.10	24.4 ±0.30	6.7 ±0.30	290 ±0.088	10.7 ±0.12

Biochemical analysis

Total Soluble Sugar (TSS) increased linearly from 2.971mgg^{-1}dwt at 0 time to 20.42 mgg^{-1}dwt. after 144 hours following harvest (Table 1). On the other hand starch content showed a slight decreasing trend from 5.36 to 4.25mgg^{-1}dwt during desiccation (Table 1). An initial decrease of total protein content from 37.6mgg^{-1}dwt to 21.9mgg^{-1}dwt after 12 hours desiccation was recorded, and subsequent desiccation did not reveal any difference in total protein content of seeds (Table 1). An increase in free amino acid content was recorded from 0 to 12 hours of desiccation, and the level retained up to 72 hours of desiccation and later a slight decrease was noticed, but was higher than the initial content (Table 1). Lipid content of fresh seeds was 305mgg^{-1}dwt and on desiccation no difference was recorded till 72 hours. At 96 and 144 hours of desiccation a slight decrease in lipid content was noticed. Total phenolic content of seeds was increased with increase in desiccation from 4.75mgg^{-1}dwt to 10.7 at 144 hours (Table 1).

Enzyme activities

Both the anti-oxidant enzymes, peroxidase and poly phenol oxidase showed high activity during desiccation. Peroxidase enzyme activity was doubled at 12 and 24 hours desiccation and later a sharp decline in the activity was recorded (Table 2). Similarly Polyphenol oxidase activity was increased from 0.039 absorbance min-1mg protien-1 to 0.120 in 12 hour and 0.138 at 24 hour. Subsequent desiccation resulted a sharp decrease in poly phenol oxidase activity (Table 2).

Table 1. Changes in Peroxidase (POD) and Polyphenol Oxidase (PPO) activities during desiccation in Hydnocarpus alpina seeds. Mean ±SD of 3 measurements.

Dessication Period (hours)	Peroxidase (POD) Δ470min^{-1}mg protein	Polyphenol Oxidase (PPO) Δ525 min^{-1}mg protein
0	0.153 ± 0.023	0.039 ± 0.01
12	0.318 ± 0.013	0.120 ± 0.02
24	0.363 ± 0.040	0.138 ± 0.012
48	0.255 ± 0.050	0. 084 ± 0.087
72	0.108 ± 0.020	0.030 ± 0.05
96	0.0.90 ± 0.021	0.027 ± 0.02
144	0. 053 ± 0.015	0.030 ± 0.01

Discussion

Seed desiccation in *Hydnocarpus alpina* as gauged by changes in fresh weight and moisture levels followed a pattern typical of recalcitrant seeds, i.e. which cannot be dried below a relatively high moisture content without damaging viability, one of the characteristics of recalcitrant seed (Roberts, 1973; Ellis, 1991). Desiccation sensitivity of the recalcitrant seeds is a major problem for long-term conservation of genetic resources. It is a complex physiological phenomenon and is likely to be mediated by a series of deleterious processes. In the present study, the desiccation resulted in a series of changes in normal cellular set up and metabolism of *H. alpina* seeds. High moisture content is an essential requirement for recalcitrant seeds to remain viable (Purohit *et al.*; 1982, Daws *et al.*; 2006). Fresh seeds of *H. alpina* have 41% moisture content with 63.3% germination. When seeds were desiccated at open laboratory conditions for 144 hours, moisture content reduced to 9.4% and resultant germination was only 8.3%. An inverse correlation recorded with desiccation and percentage of germination which was significant at 0.01% P level (Fig. 1b). Significant linear relationship was also found between germination percentage and moisture content in the seeds of *H. alpina* (Fig. 1d). Desiccation resulted in rapid loss of moisture content and the inverse relationship was significant at 0.001% P level during desiccation (Fig. 1a). High leaching of solutes was evident by the linear increase in conductivity during prolonged desiccation (significant at 0.001% P level, Fig. 1c), which may be due to the adversely affected cell wall organization of seeds. The result of the present study is in agreement with the findings of Anilkumar *et al.*, (2002) and Kamarudeen, (2003), in that the longevity of seeds reduced when their moisture content decreased.

Metabolic imbalance developed during seed desiccation may lead to viability loss (Atherton, 2000, Viji *et al.* 2013). The drying of *H. alpina* seeds ultimately might have altered the membrane integrity, which is reflected by the increased electrolyte leakage is in accordance with the works of Li and Sun, 1999. According to Bewley (1986), the electrolyte leakage increasing progressively as a consequence of seed desiccation indicates a reliable index of seed viability.

Increase of total soluble sugars during the course of seed desiccation in *H. alpina* seed is a character related to the desiccation sensitive seeds of Tea, Cocoa and Jackfruit, which showed a decline in viability and moisture level associated with increased leachate conductivity and soluble carbohydrate (Chandel *et al.*, 1995).

The levels of lipid content during desiccation in *H. alpina* seeds are consistent with the results of Abdul Baki (1969); Bewley and Black (1982). The increase in fatty acids with the progress of deterioration has been well documented in seeds. The widespread occurrence of this increase and the role of lipids in membrane structure have led some workers to suggest that viability of seed is closely associated with membrane integrity. So the lipid analysis gave a little insight into recalcitrance, although the hydrophobic nature of lipids may be a factor in cell trauma (Connor *et al.*, 2000). The studies carried out by Stewart and Bewley (1980) substantiate the result of the present study in that the level of lipids decreased with desiccation. In *H.alpina*

seeds, a slight decrease was noticed in the level of starch during desiccation in line with Bewley and Black (1982). Depletion of starch held during desiccation in recalcitrant seeds of Landolfia kirkii through histochemical studies was revealed by Berjak *et al.*, (1989). Comparing reduction of fat from stage to stage is negligible. But, when compared to other compounds like sugars and starch, reduction in lipid is significant.

Reduction in protein content is in conformity with the result of Finch-Savage *et al.*, (1996) and Chaitanya *et al.*, (2000). The decrease in protein levels was noted in the initial period of drying, resulted in the formation of free amino acids and preceded towards a non-viable seeds (Bewley and Black, 1982). In the present study a corresponding increase in amino acids level was noted during protein denaturation.

In *H. alpina* seeds, the activities of peroxidase and polyphenol-oxidase were enhanced during the initial period of drying and suppression of these activities was noted during the latter period of drying. This may be due to the loss of moisture content associated with the decrease in seed viability. Similar changes were noted in the recalcitrant seeds of *Guilfoylia monostylis* and *Telfairia occidentalis* (Nkang, 1988; Nkang *et al.*, 2000). The present study revealed the effect of desiccation in moisture content, germination and the subsequent impact on biomolecules, which in turn can be used as a marker for viability loss in recalcitrant seeds. However, an indepth analysis on the biochemical content can provide valuable information on the properties of different biomolecules and their functional behaviour during desiccation of recalcitrant seeds.

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