SEED GERMINATION AND RESTORATION STUDIES OF *HYDNOCARPUS ALPINA* WIGHT., AN ENDEMIC MEDICINALLY IMPORTANT TREE SPECIES OF WESTERN GHATS

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Abstract- ***Hydnocarpus alpina*** **Wight is an evergreen medicinal tree species endemic to Western Ghats. Narrow endemism makes this species vulnerable to extinction. Fresh seeds on harvest showed 20% moisture content with 80% germination. The viability of the seeds determined on the basis of germinated seeds. Associated with germination, different biochemical parameters such as analysis of primary metabolites like total soluble sugars, starch, amino acids, total proteins, phenols, reserve mobilites such as amylase, protease etc., were recorded. The results emphasized that, sequential loss in moisture content leads to drastic change in biochemistry resulting in the loss of seed viability and the percentage of germination. So these parameters can be considered as the markers for detecting seed viability and behaviour. Reserve mobilites are high during the germination it is helpful for the purpose of restoration.**

Keywords **- Germination, Recalcitrant, Amylase, Protease, Moisture content.**

INTRODUCTION

The Western Ghats, also known as the Sahyadri Hills, are well known for their endemism. Recently, the area is facing severe threat and endangerment due to various physical and physiological reasons. Some plants are found only in Western Ghats. Identification of those plant species and their conservation, restoration is equal to the conservation of the whole plant population.

Hydnocarpus alpina Wight is an evergreen endangered medicinal tree species endemic to Western Ghats. Several local names are available based on the distribution among the state in India that is Torathi (Kannada), Maravetti/Marotti (Malayalam), Attuchankalai (Tamil), Kastel (Hindi). This tree has very appreciated value in various medicinal properties like anti-larvicidal, anti-feedant, antimicrobial etc., due to the presence of significant chemical constituents (1).

Trees of *H. alpina* grow up to 8 m tall, Flowers unisexual, solitary and white in colour. Fruits and seeds are berry, seeds numerous. Flowering and fruiting in between February–July. The chemical constituents found in this species are chaulmoogric acid, hydnocarpic acid, apigenin, hydnocarpin, fixed oils, tannins etc. Its timber is good for construction purposes and is used for Beams and Rafters. The seeds contain high level of fatty oil which is similar to chaulmoogra oil used extensively in the treatment of leprosy and other cutaneous diseases and also used as an illuminant (2).

Seeds are heterogeneous storage reserves with wide array of storage compounds that include various soluble carbohydrates, starch polymer, storage proteins and lipids. Seed systems biology remains an enigmatic subject in understanding seed storage processes, maturation and pre germinative metabolism. *Hydnocarpus alpina* is a tree with recalcitrant type seed (3). So the germination studies including biochemical assay and analysis of reserve mobilites are utmost important for the conservation and restoration of this rare endemic medicinal tree species.

MATERIALS AND METHODS

Moisture content analysis

The seeds of *Hydnocarpus alpina* are recalcitrant in nature and lose their viability within a few days after detachment from the mother plant. The mature fruits of *H. alpina* were collected from Thiruvananthapuram and Kollam Districts. The fruits initiation starts during the months of January-February. But maturity attain after 5-6 months later ie., during June - July. Seeds collected from the ground through its natural shedding. De-pulped seeds are then used for further studies.

Moisture content was determined by the difference between fresh and dry weight. For dry weight determination, the seed material was taken in a pre - weighed bottle and weighed in an electronic balance, dried in hot air oven at 103^0 C for 17 hours (until the constant weight were obtained) - Low Constant Air Oven Method (4)

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 (5). Seed germination test was carried out in three replicates of 50 seeds each, rolled in acid free germination paper kept in seed germinator (KEMI) 28 ± 2º C and 85% RH.

Biochemical extraction and estimation

One gram fresh tissue sample was homogenized in 80% ethanol. Homogenate was then centrifuged at 3000 rpm 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 (6) and amino acid (7). 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 (8). The left over residue was ground with 30 and 15% Perchloric Acid (PCA) respectively at two times,

centrifuged at 3000 rpm for 10 minutes each and combined supernatant used for the estimation of starch (9). For protein estimation, the tissue was homogenized in double distilled water and precipitated with Trichloro Acetic acid (TCA) and estimated following (10).

Extraction of enzymes

α – amylase, β – amylase and protease

Amylase assay (α -1,4 glucan4–glucano hydrolase E.C. and α – 1,4 – glucan malto hydrolase E.C)

Amylase activity was assayed following the procedure described by (11).

Enzyme extraction

Weighed (1g) plant material is homogenised with 0.1M Sodium Acetate Buffer ($_P$H 4.7).The homogenate was centrifuged at 5000 rpm for 30 minutes at 4^0C.After decanting the supernatant, the extraction was repeated twice with the homogenising buffer. The combined supernatant served as the enzyme source for α and β-amylase.

a) Total enzyme assay (α amylase and β amylase)

The reaction mixture contained 1ml of 1% starch in sodium acetate buffer (0.1M, pH 4.7) and 1 ml of properly diluted enzyme source and incubated for 15 minutes. The enzyme reaction was stopped by the addition of 2 ml of dinitro salicylic acid (1% prepared with 0.2% crystalline phenol, 0.05 % sodium sulphite in 1% NaOH). The mixture was heated in a boiling water bath for 5 minutes, and 1 ml of 40 % sodium potassium tartrate solution was added. The tube was cooled and the mixture was made to 10 ml by adding distilled water. Orange red colour formed (nitro amino salicylic acid) was read at 560 nm. The control was prepared by adding di nitro salicylic acid before incubation. The amylase activity was expressed as maltose released 15 min $^{-1}$ mg protein $^{-1}$

b) α-amylase assay

Before adding enzyme to the incubation mixture, the activity of β amylase was completely eliminated by heating the enzyme extract at 70^0C for 5 minutes resulting only α-amylase. From the total amylase activity, the α–amylase activity was subtracted and the β-amylase activity was calculated.

ii) Protease assay

Protease activity was measured by the amount of proteins converted into amino acid as compared to a control. This was followed by the method (12).

Enzyme extraction

Tissue samples were homogenised in a pre-chilled mortar and pestle with 5.0 ml (0.1 M) ice cold sodium phosphate buffer (pH 7.0). The homogenate was centrifuged at 3000 rpm for 10 minutes and the supernatant was collected. The residue was again washed thrice with known volume of extraction buffer and the combined supernatant served as the source for protease assay. All the enzyme extractions are carried out at 4^0C.

Enzyme assay

The reaction mixture contained 1ml of 1% casein in sodium phosphate buffer (0.1M and pH 7.0) and 1 ml of properly diluted enzyme source in a test tube and incubated at 37^0C for 1 hour. To this 1 ml of 12 % TCA was added for protein precipitation. The mixture was cooled at 3000 rpm for 5 minutes and the supernatant decanted and their protein estimated by (10). The control was prepared by adding 1ml of 12 % TCA before incubation

Statistical analysis

The data from different experiments were analysed following one way Analysis of Variance (ANOVA) and the ratio obtained were checked for significance at 1 and 5 % probability (P) level. From this calculated ANOVA the means of each treatment were separated following the least significance Difference (LSD) by Duncan's multiple range test at 1 and 5% level.

RESULTS

Germination response of *Hydnocarpus alpina*

Moisture content and germination

Fresh seeds of *H. alpina* on harvest had 20.1% moisture content. Seed moisture content and final germination percentage decreased considerably. Fresh seeds of the species showed 80% germination. After 8 days, the moisture content dipped to 17%, considered as Critical Moisture Content and the germination was reduced to 25%. (Table-1)

Table 1: Germination response of *Hydnocarpus alpina* seeds

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Desiccation periods (Days)	Moisture content (%)± SE	Germination (%)± SE	Days required for germination (Germination energy)
1	**20.1 ± 0.31*****	**80 ±0.39*****	14
4	**19.3 ±0.12*****	**75 ±0.33*****	18
8	**17.6 ± 0.12*****	**50 ±0.34*****	**25**
12	**13.6 ± 0.08*****	**40 ±0.34*****	28

SE: Standard Error of the Mean

Biochemical studies

During the germination process the TSS content in the seeds of *H. alpina* gradually increased during each stages of imbibition. During the initial stage ie, at 0 days of imbibition the cotyledon contains 10.46 mg /gm dwt of sugars. When the imbibition progresses, the Total Sugar Content (TSS) decreases in cotyledon. Subsequent stages of germination, the TSS content varying and mobilization occurs at the root, plumule and cotyledons (Table-2). The starch content in *H. alpina* seeds is high at the initial imbibition periods. During each stages of imbibition, the starch content decreases and it also mobilizes in to the radicle and plumule. As the imbibition period increases the content of starch declines (Table-2).The phenol content was maximum at the initial stages of germination. Then the content of phenol mobilizes to radicle and plumule, but not at a significant level (Table-2). Amino acid content is less at the initial stages of imbibition. Later the content declines at each stages of germination. Amino acid content also mobilizes in to the radicle and plumule as well as the cotyledon (Table-2).

Table 2: Reserve mobilization during germination of *H. alpina* seeds.

± SE: Standard Error of the Mean

Imbibition periods (days)		Total soluble sugars (mg g-1 d.wt. ±SE)	Starch (mg g-1 d.wt.±SE)	Total phenol (mg g-1 d.wt.±SE)	Protein (mg g-1 d.wt.±SE)	Amino acid (mg g-1 d.wt.±SE)
0	Cot	10.46 ± 0.05*	83.01± 0.06**	4.27 ±0.39**	39.14 ±0.74*	0.210 ±0.061*
10	Cot	6.49 ± 0.04**	41.21 ±0.05**	2.21 ±0.21**	19.57 ±0.96**	0.048 ± 0.0012***
	Rad 3 cm	4.23 ± 0.38**	39.26 ±0.22**	2.05 ±0.10*	18.71 ±0.81**	0.037 ± 0.001***
20	Cot	2.83 ± 0.12*	21.85 ±0.78*	1.165±0.16**	18.00 ± 0.81*	0.03 ± 0.008***
	Rad 4.5 cm	2.130.± 21*	20.05 ± 0.7*	1.01 ± 0.17*	17.00 ± 0.17*	0.025 ± 0.07***
30	Cot	8.17 ± 0.43*	76.1 ± 1.65*	2.48 ±0.25*	25.4 ± 0.98*	0.114 ± 0.002***
	Root	7.0 ± 0.00*	39.71 ±7.66*	6.0 ± 1.00*	21.62 ±0.76*	0.138 ± 0.001***
	Plumule	3.46 ±0.29*	3.14 ± 0.56	2.33 ±0.33	14.86 ±0.72	0.111 ± 0.002***

Enzyme assay

Amylase activity

Amylase is a catabolic enzyme whose activity is very high during the initial period of germination in the seeds of *H. alpina.* Activity of amylase increases gradually as the imbibition progresses. Even after 30 days the conversion of stored metabolites shows similar trend and it occurs in to the root, cotyledon and plumule. (Table-4)

Table: 4 –Activity of the enzyme amylase

Imbibition (days)		Amylase (mg/g.fwt.)
0	Cot (Radicle 0.5 cm)	0.230 ±0.005
10	Cot	0.140 ± 0.07**
	Radicle 3cm	0.127 ± 0.03**
20	Cot	0.028 ± 0.001***
	Radicle 4.5 cm	0.031 ± 0.002***
30	Cotyledon	0.143 ± 0.045***
	Plumule	0.060 ±0.005***
	Root	0.004 ±0.005***

± SE: Standard Error of the Mean

Protease activity

Protease is also a catabolic enzyme like amylase. The content of protease in H. *alpina* showed similar trend as amylase (Table-5).

Table 5: Activity of the enzyme protease

Imbibition (days)		Protease (mg/g.fwt.)
0 days	Cot	**0.759 ± 0.006*** **
10 days	Cot	0.435 ± 0.261*
	Radicle 3 cm	0.421 ± 0.252*
20 days	Cot	0.354 ± 0.126*
	Radicle 4.5 cm	0.318 ± 0.114**
30 days	Cot	0.401 ± 0.217
	Radicle 6cm	0.428 ± 0.221
40 days	Cot	0.708 ± 0.160*

	Plumule	0.753 ± 0.021***
	Root	0.675 ± 0.002***

± SE: Standard Error of the Mean

Seedlings for restoration practices

Acid free germination paper and riverine soil gives maximum amount of seedlings for restoration purposes.

DISCUSSION

Germination incorporates those events that commence with the uptake of water by the quiescent dry seed and terminate with the elongation of the embryonic axis (13). The visible sign of germination is the penetration of radicle of the embryo through the surrounding tissues; the result is often called visible germination (14). In *H. alpina*, the plumule emergence observed after 72 hours. The root, stem and leaf emergence were observed after 20 days of imbibition. Germination is an energy requiring process and is therefore dependent on respiration of the seeds. The reserve mobilisation during the germination of *H. alpina* seeds is in agreement with the findings of *Hopea parviflora* and *Vateria indica* (15). Upon imbibition, the quiescent dry seed rapidly resumes metabolic activity. The result showed that the starch content of *H. alpina* seeds is high at their initial stages of germination, and then it degraded and mobilizes to the embryonal axis from the cotyledons. The starch content in *Citrullus lanatus* seeds was positively correlated with germination rate but not with the final germination percentage (16). The data on soluble sugar (such as glucose and sucrose) was positively correlated with germination percentage of *Medicago truncatula* seeds (17).

The phenol content is comparatively low with other biomolecules during imbibition. Increased total phenol content in fresh seeds may be the chemical defences associated with seed germination. Phenol content decreases in the cotyledons, possibly due to accelerate germination processes. Once the again the phenol content increase in the cotyledons and roots as they mature to form intact plant parts.

Plant seeds store a large amount of proteins, lipids and carbohydrate to enable germination and subsequent development in to mature plant. The existence of lipids as a major reserve food in some seeds indicates its importance as a fuel and biosynthetic precursor. During the imbibition and germination of seeds, free fatty acids are released by lipase mediated triacylglycerol breakdown (18 &19). Accumulated macromolecules in seeds are used as an energy source for early seedling development and seed germination. Detoxification of ammonia in higher plants is usually considered to occur by way of the acid amides (20). The amino acid level of *H. alpina* seeds during imbibition showed almost increasing trend throughout the period.

Amylases are the enzymes that convert or breakdown starch into glucose (21). They are the most important carbohydrate degrading enzymes produced by microorganisms, animals and plants. Germinating seeds generally exhibit high amylase and protease activities. This is because; these enzymes are synthesized during seed germination to mobilize stored food (starch and protein) for the survival of the young plant until it is capable of making its food by photosynthesis (22).

In the present study it is found that the progressive increase of amylase activities during initial period of imbibition (0-72 hours) in *H. alpina* promotes the germination processes with the breakdown of starch reserves. (23) Showed that both specific and total alpha amylase activity increased in cotyledons of germinating peas during the first 10 days of germination. Most of the increase occurred after day 5 and was more dramatic in seeds germinated in the dark.

In the present study, the activity of amylase was slightly low during 0 days of imbibition. Upon the absorption of water by dry seeds activity of amylase increases. From 0 days (24 hours of imbibition) to 10 days of imbibition the activity of amylase increases gradually and the content mobilizes into radicle and plumule also. The increased activity of α-amylase during germination is probably due to the de novo synthesis in these cases (24). Maximum amylase activity was observed during initial periods of imbibition.

It was seen that protease activity present in dry seed is enhanced during germination. The combined action of various proteolytic enzymes thus results in total degradation of storage proteins (25) and the products are made available for the germination and seedling establishment.

The study revealed that amylase and protease are the catabolic enzymes whose activity is very high during the initial period of germination (26). From 0 days of imbibition, the activity of amylase and protease increases gradually. The activity of protease also like that of the amylase ie, at initial stages it was very high. These findings are in line with the results of (27) observed in germinating Beans ie., in germinating beans the proteolytic activity increased during the first 7 days of germination. Even after 30 days, the germinated Hydnocarpus seeds showed the mobilisation and conversion of stored metabolites in to the root, cotyledon and plumule respectively. The free radical scavenging enzymes doesn't show any significant variation during imbibition.

The maximum percentage of germination was recorded in acid free germination paper and riverine soil. So the restoration practices suggests the above method for more number of seedling propagation

The present study again reveals the nature of recalcitrant seed. This results correlate with the works of previous findings of (1) and (28). Seed is the only propagation tool for most tropical tree species. So germination of maximum seedlings and their restoration is utmost important. To know the germinative mechanisms we should know the biochemical characters behind them. This study helps us to improve the knowledge about seed germination mechanisms thereby rise proper conservation strategies.

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