RESEARCH ARTICLE

Viability loss of *Dendrocalamus hamiltonii* seeds with storage associated with membrane phase behaviour and hormonal analysis

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ABSTRACT

Bamboo seeds are scarcely available and also they have a very short viability of 2-3 months as they flower after a long interval and generally flowering is infrequent. Generally they are stored under controlled condition of 4 degree Celsius in desiccators under anhydrous calcium chloride to maintain viability for longer period of time. During storage upto 18 months, deterioration, increases with ageing. Biological membranes with a normal composition and organisation regulate the transport of material into and out of the cell. Damage to the organisation of cell membrane during seed ageing may constitute an important factor in explaining seed deterioration**.** The damage caused to membrane through deterioration that provides lower selectivity and hence increase in the leakage of solutes to the environment is one of the main cause of the decline in the physiological quality of seeds i.e germination studies and hormonal studies. Effect of hormone were studied i.e IAA, Gibberellins and Abscissic Acid after every 6 months upto 18 months period. One of the major changes during seed storage is membrane deterioration, which leads to the loss of seed viability. The aim of the study is to correlate membrane integrity deterioration with seed viability or physiology during storage upto 18 months in species of seeds of *Dendrocalamus hamiltonii.*

Keywords: Deterioration, physiology, membrane integrity, seed viability, storage

INTRODUCTION

The importance of bamboo as an ecofriendly raw material, capable of meeting multifarious needs of the people at large, is gaining global acceptance. From a "poor's man timber",bamboo has now been elevated to the status of "the timber of the 21st century". Bamboos provide us the three basic necessities of life *i.e.* food, shelter and clothing. They are put to over more than 1500 different uses. Infact, no other plant benefits us in so many ways as bamboos. As the seeds are stored, they surely

undergo ageing during which symptoms such as reduced rate of germination seed vigour, and greater susceptibility to attacks by microorganisms are observed. Though the causes of deterioration of seed viability during storage has not been fully understood, scientists relate it to bioenergetics disturbance (Ching, 1982), damage to nucleic acids (Cheah and Osborne, 1978), loss of vitamins and hormones (Copeland, 2001, Bewley and Black,1982; Richa *et al.,*2000; 2006), and membrane deterioration(Wilson and McDonald, 1986; Richa *et al*., 2006; 2010). Biological membranes with a normal composition and organisation regulate the transport of material into and out of the cell. They play a key role in maintaining seed viability and vigour. Seed viability was studied with TTC test and germination studies and their effects with ageing. Solute leakage include seed imbibitions during the process of membrane reorganization following rehydration. The rate of leakage depends on the degree of cell membrane damage and repair in response to ageing. Electrolyte leakage and electrical conductivity was studied in all the three species to find the effect of viability with membrane integrity. Electrical conductivity measurements of seed leacheates are routinely used to determine seed vigour in a number of species (Pandey, 1992). Leakage of sugars is considered as a less reliable index of membrane integrity than the leakage of electrolytes. Membrane damage that occurs during seed storage contributes to a loss of viability and vigour. Ultrastructural studies on seeds of three species of bamboo was done to distinguish between the fresh seeds and aged seeds of one year. Ultrastructure studies were done to distinguish between dry and imbibed seed tissues of soybean(Chabot and Leopold, 1985).Chabot and Leopold (1985) studied characteristics of the other organelles and cells under stress of chilling injury in soybean used electronic microscopic (EM) studies (Chabot and Leopold, 1985). Most studies have focused on storage tissues such as cotyledons in soybean (Chabot and Leopold, 1985). Studies on soybean seed anatomy using transmission electron microscopy (TEM) featured testa and the phloem and xylem of the vascular sutures in the soybean pod. It has been observed that protein oxidation can cause modification of amino acid side chains, backbone fragmentation, protein dimerization or aggregation, and the unfolding or altered conformation of proteins(Hawkins and Davies, 2001). Kobayashi *et al*.(1989) analysed the endogenous level of gibberellins in shoots and ears of two dwarf rice (*Oryza sativa*) cultivars. Their results indicated that GA_1 is the active gibberellins that regulates vegetative growth of rice. The endogenous level of GA_4in the ears of the two dwarf cultivars affect GA_4 in vegetative growth of rice. A 5-fold decline in the level of GA_{19} and a considerable increase was observed in the level of GA_{20} and GA_{29}, under long- day treatment. These results were consistent with the hypothesis that GA_{19} is converted to GA_{20} during stem growth and that this conversion is under photoperiodic control. Application of GA stimulates dormant seeds of many species to germinate .In *Emblica officinalis* seeds treated with GA(100 ppm) has higher germination percentage and number of secondary roots were reported by Wagh *et al*. (1998). Richa and Sharma (1994) reported that 5 ppm concentration of GA_3 stimulated germination in stored seeds of the bamboo *Thrysostachys siamensis* to 86.6% and 10 ppm GA_3 to 50% in *Dendrocalamus strictus.*

MATERIAL AND METHODS

Prior to germination studies the seeds were surface sterilized by soaking them in 0.5% mercuric chloride ($HgCl_2$) for two minutes followed by thorough washing in running water. They were later rinsed with distilled water 2-3 times. Ten randomly selected surface sterilized seeds were placed equidistantly in pre-sterilized petridishes (Ø 9.0 cm) lined with filter paper. One set of seeds were placed in petridishes, on filter paper soaked in distilled water as control. The entire experiment was conducted in laboratory condition in seed germinator where temperature was maintained at 28°C ± 2°C. The seeds were observed daily and the number of seeds germinated and their respective root and shoot length were recorded for 14 days. The experiment was repeated after regular intervals of six months for upto one and a half years.

Following parameters were studied for seed germination and growth kinetics.

Germination percentage (G%): Emergence of radicle was considered as an indicator of germination. Number of seeds germinated was noted after every 24 hrs for 14 days.

$$\text{Germination Percentage (\%)} = \frac{\text{Total number of germinated seeds}}{\text{Total No. of seeds sown}} \times 100$$

Vigour index (VI) (Abdul- Baki and Anderson, 1973)
Vigour index was calculated by the following formula

VI=G % X Root Length (cms)

TTC(2,3,5- triphenyltetrazolium chloride)Test for viability (Steponuks and Lanphear, 1967) was done . The seeds were taken in 3 replicates with 10 seeds each. All the replicates were placed in small glass tubes containing 3ml of 0.1M K_2HPO_4-KH_2PO_4 buffer (pH-7) with 0.5% (w/v) TTC. The ovules were incubated for 20 hrs at 28°C in darkness. The TTC solution was drained. The seeds were washed twice with distilled water and 2ml of ethanol was added. The tubes were kept in a water bath at 95°C until complete evaporation of the ethanol. 4ml of ethanol was added again and tubes were vigourously shaken. The absorbance due the formazon formation was recorded at 520nm against ethanol.

Membrane integrity was studied by Duke and Kenyon, 1993 method. In 10 ml of distilled water, 20 seeds were added. After two hours, measure electrical conductivity. Boil it for 5 minutes and then cool it. Then measure the electrical conductivity.

The Electrolyte leakage measurements of seed leachates was done when10 seeds of each species were placed in 20 ml of distilled water for 12, 24 and 48 hrs each, in conical flasks which were covered with aluminium foil to reduce evaporation and dust contamination and maintained at 20°C ±2. The solutions were filtered and final volume was made to 20ml. The medium was gently swirled for 10-15 seconds and the conductivity was measured using conductivity meter. These measurements were recorded at regular intervals of six months for one and a half years.

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Transmission electron microscope (TEM) study of seeds of three species was carried out to compare the seed structure (morphological) of viable and non-viable seeds. Following procedure was followed.

a. Surface cleaning: Seeds were crushed to form powder and a suspension of lyophilized powdered seed was prepared in double distilled water.
b. Fixation and dehydration: Few drops of the suspension were deposited into a carbon-coated copper grid and immobilized for 1-2 min. After immoblization the excess solution was wicked off with filter paper and sodium phosphotungstate solution (0.2%, w/v) was added for negative staining. The grids were washed with double distilled water twice and dried after the few seconds.
c. Observation: The morphology of NPs was observed using Philips CM-10 transmission electron microscope (TEM) (FEI, USA) using an acceleration voltage of 200 kV.

Endogenous levels of following plant growth hormones were estimated in seeds after regular intervals of six months. Prior to extraction ,seed were imbibed in distilled water for 12 hrs.

Auxin(Nagar, 1996)
5 gm fresh weight of seeds was separately homogenized in chilled 80% methanol three times. The homogenates were pooled and centrifuged at 10000 rpm at 5°C for 30 min. The supernatants were concentrated in vacuum at 30°C and then applied to Polyvinyl-prryrolidone (pvp) columns. Elution was carried out by a gradient of 30-70 % methanol and finally with 100% gradient of methanol for 15 min. at a flow rate of 1ml/min. The column evaluated was passed through an ultraviolet (UV) detector at 254 nm, and IAA was measured by referring to an authentic standard of indole-3-acetic acid.

Gibberellins (Chen Wen-shaw, 1994)
The endogenous level of GA was determined by the method given by Chen Wen- shaw (1994) with a few modifications.The equivalent of 3 gm dry weight of bamboo seeds was homogenized and extracted three times with 80% (V/V) methanol (500 ml). The methanol extract was concentrated in vacuum and the residue was diluted with H_2O to 2 ml mixed 0.5 gm Celite, dried in a gentle air stream, and loaded onto a SiO_2 partition column (prepared from 5 gm of deactivated woelm SiO_2 slurried in 95/5 V/V ethyl acetate n-hexane). This was first eluted with 80 ml ethyl acetate /hexane (95/5V/V) to remove free Gas and then with 100% methanol (150 ml) to remove highly water soluble GAs and GA conjugates. The Ethyl acetate/hexane elute was neutralized with 0.1 N NH_4OH and then dried in vacuum and chromotographed on Lichrosorb RP 18 (10 µm) HPLC column (250X44.6, ID) eluting with a linear gradient of methanol (30%100%) in 1% aqueous acetic acid, run in 25 min. flow rate 2.0 ml/min.

Abscissic acid(ABA)(Nagar, 1996)

Each seed sample of 5 gm freshly homogenized in chilled 80% methanol (20 ml/g) containing butylated hydroxytoluene (BHT) at 100 mg/l. The homogenates were stored for 24 hrs in the dark at 4°C and then filtered in vacuum.The residues were re-extracted five times with chilled methanol,filtered and centrifuged at 10,000 rpm at 5° C. The supernatants were dried in vacuum,dissolved in 2 ml of 0.1 M potassium phosphate buffer (pH-8.8) and then applied to polyvinyl- pyrrolidone (PVP) columns. The column elutes were passed through an UV detector at 254 nm and ABA was measured by referring to an authentic standard of ABA (Sigma Chemical Co. St/ Louis, USA)

Statistical analysis

Data were statistically analyzed using the software spss 14. Analysis of variance (ANOVA) was used to test the significance of variance sources, while LSD test (p= 0.05) was used to compare the difference among treatment means.

RESULTS AND DISCUSSION

The seed viability study includes germination percentage and TTC Test after period of 18 months of storage. Germination percentage was maximum *in Dendrocalamus hamiltonii* i.e 81.2% which reduced to 20% after 6 months of storage and after 12 months viability reduced to zero after 12 months. Triphenyl tetrazolium chloride (TTC) activity denotes the cellular respiration of seeds. The TTC test has been widely used to evaluate seed viability and vigour. The TTC and the EC tests are accepted as method of testing viability by the International rules for seed testing (ISTA, 1999)although the TTC test is the most widely useful. This method has been successfully used to predict germination and seedling growth of a wide range of species as well as viability (Wang *et al.,* 2008). Seed viability of seeds of three species presently evaluated by TTC method showed that the TTC activity reduced with increased in time period.

Table 1: Germination parameters in seeds of three species at 6 monthly ageing intervals in natural condition seeds

Species	Freshly procured			6- month aged			12- month aged		
	G%	V.I	E.I	G%	V.I	E.I	G%	V.I	E.I
DH	81.2*	1236.4*	3.5	20*	114.2	1.4	0	0	0

Table 2: Electrolyte leakage (m/ mhos) in seeds of three species at 6 monthly natural ageing intervals

Species	Freshly procured	6 –month aged	12 –month aged	18 –month aged
DH	115*	136*	159*	199*

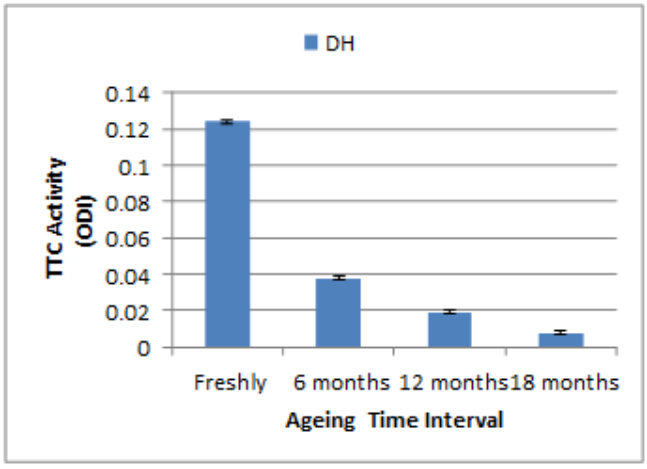

Fig. 1: TTC activity in seeds of three species at 6 monthly naturally ageing intervals
(Bars represent standard errors at 5% level)

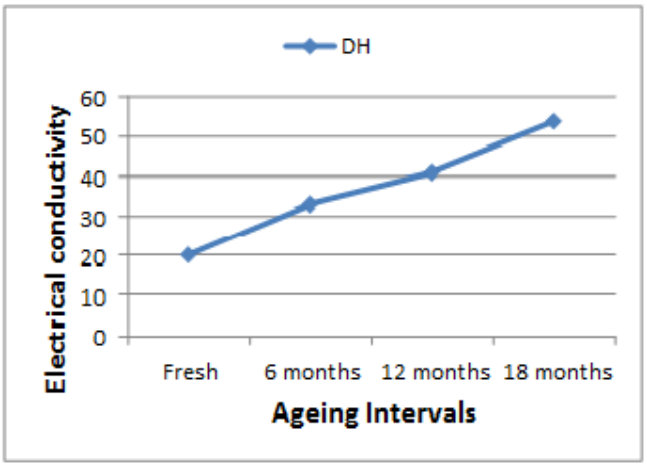

Fig 2: Electrical conductivity in seeds of three species at 6 monthly naturally ageing intervals

Germination studies indicated maximum germination percentage in case of *Dendrocalamus hamiltonii* which reduced to 20% after 6 months of ageing.

Electrical conductivity in seeds of three species at 6 monthly naturally ageing intervals. Electrical conductivity was maximum in *Bambusa bambos* out of all the three species which reduces with ageing and minimum in *Dendrocalamus hamiltonii.* Measure of electrical conductivity is a good indicator of viability of seeds. The loss of germinability with ageing coincides with a considerable increase in membrane permeability of the embryo-axis cells. The biochemical observations revealed a concomitant decrease in antioxidant potential and an increase in solute leakages which is evidenced from elevated electrical conductivity,These results substantiate that membranes are damaged by increased free radical attack during artificial ageing of seeds.
Seeds under natural ageing after 6- months of ageing, electrolyte leakage was recorded as 136, 138 and 145 respectively. At 12- months of ageing, electrolyte leakage was recorded as 159, 166 and 168 in *D. hamiltonii, D. strictus, B. bambos* respectively whereas at 18- months of ageing electrolyte leakage became 191, 250 and 298 respectively in the seeds of three species. Electrolyte leakage was also maximum in *Dendrocalamus hamiltonii*.

TEM study was made to study morphological feature of the cells of seed coat structure of fresh viable and non-viable aged bamboo seeds of *D. hamiltonii* species. Freshly procured seeds showed compact membrane structure in all the three species. The seed showed degradation of surface after one year. The present study indicates that seed coat structure is a reliable factor for correlating seed viability with ageing and it can be detected by observing changes produced in the seed coat membrane. TEM observations of seed coat were focused on the arrangement and characterization of cells and characters of interspace. The freshly procured seeds showed compact structure as compared to non-viable seeds which showed broken structure. The seeds showed degradation of structure with ageing after one year. During germination, seed coat break open and provide components that contribute to biotic and abiotic stress resistance. Microscopic level of study has been found to be of great help in determining the relationship of ageing and membrane degradation. Seed coat protects the embryo during dormancy and maintains an environment around the embryo that is conducive for quiescence. During germination, the seed coat must weaken and break open and provide components that contribute to biotic and abiotic stress resistance. Several earlier studies on seed coat morphology by election microscope have been found of great help in determining the relationship of ageing and membrane deterioration. (Zeng *et al*., 2004; Moise *et al*, 2005). Scanning electron microscopy has been particularly useful in studying seed coat with ageing and imbibitional damage has been linked to the loss of membrane barrier properties (Golovina *et al*. 1997; Gibson, 2004).

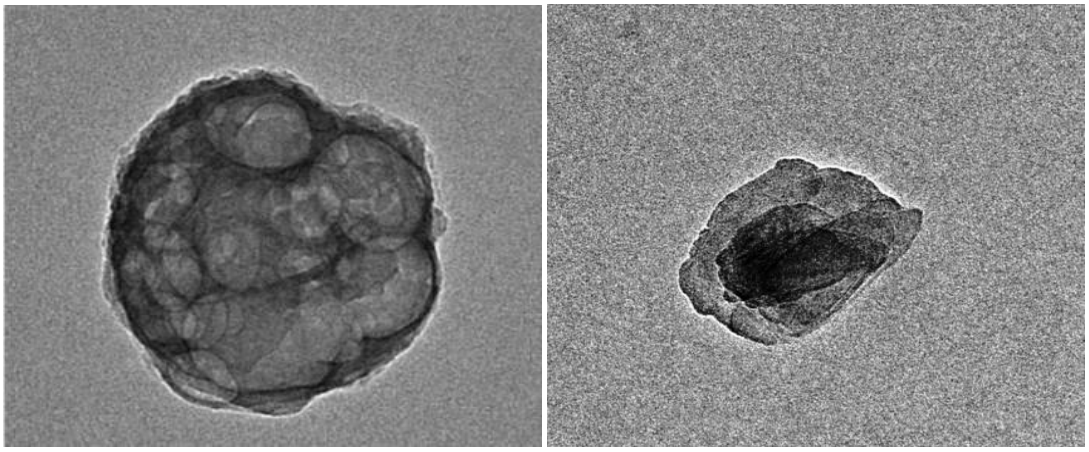

Fig. 3: Transmission Electron Microscopic (TEM) observations of seed coat
Transmission Electron microscopic photographs showing fresh (Left) and non viable (Right) seed membrane taken after one year of *Dendrocalamus hamiltonii*.

Table 3: Endogenous IAA levels (expressed as µg/gmfwt.) in seeds at 6 monthly stages of ageing

Species	Freshly procured	6-month aged	12-month aged	18-month aged
		Natural condition	Natural condition	Natural condition
DH	12.34	2.34	0.78	0.12

Table 4: Endogenous GA_3 levels (expressed as µg/gmfwt) in seeds at 6 monthly stages of ageing

Species	Freshly procured	6-month aged	12-month aged	18-month aged
		Natural condition	Natural condition	Natural condition
DH	67.82	6.78	0.99	0.30

Table 5: Endogenous ABA levels (expressed as µg/gmfwt) in seeds at 6 monthly stages of ageing

Species	Freshly procured	6 -month aged	12-month aged	18-month aged
		Natural condition	Natural condition	Natural condition
DH	59.82	169.8	218.99	265.30

In the first two cases level of auxins and gibberellins decreases with ageing while abscissic acid level increases with ageing suggesting ageing increases the level of inhibitors which leads to deterioration of seed and reduced seed viability. The freshly procured seeds have high germination percentage, low electrical conductivity and electrolyte leakage, high formazon formation, and good membrane structure. These seeds when stored under natural conditions, show decline in germination percentage and vigour index, lower membrane integrity, higher electrical conductivity and electrolyte leakage, lower viability (TTC test) and endogenous growth regulators *i.e* IAA and GA and increase in inhibitors *i.e* ABA with the passage of time. Like all other seeds, bamboo seeds also undergo physiological as well as biochemical deterioration and this age-related deterioration also brings about membrane damage, leakage of reserve food material and depletion of optimum level of endogenous growth regulators. This deterioration can be somewhat slowed when seeds are stored under controlled conditions of temperature and humidity by storing in desiccator at 4° C under anhydrous calcium chloride.

CONCLUSION

It can be concluded that deterioration of bamboo seeds is mainly due to membrane degradation and physiology of seeds are dependent upon membrane organization which deteriorates with ageing. So the viability of seed could be retained for longer period of time by preserving its membrane degradation. It was observed that there is direct correlation between viability and concentration of growth regulators. There is a decrease in the concentration of growth promoters (IAA and GA) and increase in growth inhibitor (ABA) during seed storage

Conflicts of interest: The authors stated that no conflicts of interest.

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