Changes in Total Protein and Protease Activity in Dehydrating Recalcitrant Sal (*Shorea robusta*) Seeds

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A rapid loss of viability was recorded in sal seeds when dehydrated below 36.7 % moisture content, at ambient conditions. Seed becomes non-viable at 6 days after harvest (dah). Gradual decline in total protein content due to corresponding increase in protease activity preceded loss of viability. Almost 43.7 % and 52.6 % loss in total protein content was observed on 3 dah in embryonic axis and cotyledon respectively of seeds showing 100 % viability. No protease activity was detected in the embryonic axis and cotyledon of freshly harvested viable seeds (0 dah). The protease activity was detected after 12 hrs of storage and increased sharply with peak levels on 6 dah (0.71 ± 0.04 / min/mg protein) and 4 dah (0.16 ± 0.01 / min/mg protein) in embryonic axis and cotyledon respectively. Later on the enzyme activity declined sharply in both the tissues. Enhanced protease activity in embryonic axes and cotyledons has been discussed with corresponding decline in total protein during desiccation induced loss of viability in sal seeds.

Keywords recalcitrant, viability, total protein, protease, *Shorea robusta*
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1 Introduction

Sal (*Shorea robusta* Gaertn f.) seeds are tropical recalcitrant with germination falling dramatically when drying reduces the seed moisture content to less than about 30–40 % fresh weight basis (Nautiyal and Purohit 1985, Tompsett 1985, Chaitanya and Naithani 1994, 1998). The deterioration of seeds during dry storage is a complex phenomenon involving changes in many seed components (Priestley 1986). Membrane has been shown to be a key site of injury during seed ageing majorily due to oxidation and damage of its components, lipids and protein (Sun and

Leopold 1995). In a previous publication, we have reported rapid enhancement in superoxide mediated lipid peroxidation due to absolute impairment of scavenging enzyme (SOD) during loss of viability and suggested that membranes are the key site of injury in naturally aging desiccation-sensitive sal seeds (Chaitanya and Naithani 1998).

Net loss of protein in deteriorating seeds is perhaps the most basic of all aging related events, as these changes could underlie all other aspects of metabolic decline. The decline in protein content in aging seeds may be either due to (i) extensive damage of protein synthesizing system reported in various crop (Anderson 1970, Hallman et al. 1973) and tree (Szczotka 1975, Espindola et al. 1994) seeds of reduced germinability or non-viable seeds or (ii) synthesis or activation of large quantities of proteolytic enzymes during seed deterioration (Bewley and Black 1982).

The enhanced protease activity results in concomitant decline in storage (Ashton 1976, Misra and Kar 1990) and enzymatic protein (Perl et al. 1978). The embryonic axis, through the production of plant hormones, controls the protein breakdown by regulating the synthesis of proteases in storage tissues of *Cucurbita maxima* (Penner and Ashton 1967), *Vigna radiata* (Sze and Ashton 1971, Kern and Chrispeels 1978) and *Phaseolus vulgaris* (Gepstein and Ilani 1980).

The objective of this research was to follow changes in protein and protease activity in dessicating recalcitrant sal seeds. The relative significance and contribution of axes and cotyledon was determined during loss of viability by monitoring changes in protein content and protease activity in these tissues.

2 Materials and Methods

2.1 Collection of Seed

In this study, seeds were collected from Gariyabandh forest. The forest is situated 90 kms away to the northeast of Raipur and lies between 20°38'N latitude and 82°04'E longitude. Nearly 25–30 plus trees in the forest were marked for collection of fruits and seeds. Fully mature seeds (showing 100 % germination within 40–48 h) of sal, 63 days after anthesis, were collected and brought to the laboratory within 4–5 h (Chaitanya and Naithani 1994).

2.2 Seed Morphology

The mature fruit of sal is composed of 23 % wing, 30 % pod and 47 % kernel. The dried wings are very brittle and can easily be pulled off. The dewinged seeds contain an outer thin, brittle seed pod. Slight pressure of the fingers breaks the pod very easily exposing a rather hard ovoid kernel. In the beginning of the fruiting season, the kernel is invariably green but turns tan and finally deep brown on storage.

2.3 Seed Storage

The sal seeds were stored after removal of the dried wings (calyx) in perforated trays in two layers at ambient conditions (Temperature 32–36 °C, RH 35–45 %). Seed samples were removed at regular intervals to conduct various analyses.

2.4 Moisture Content

The moisture content of the seeds, embryonic axes and cotyledons were estimated on fresh weight basis. Three replicates of 15 seeds each of axes and cotyledons were taken and the fresh weight was determined. The dry weight of the seed was determined after drying at 80 °C for 72 h. The moisture content of the seed, embryonic axes and cotyledons were estimated as percentages water of their fresh weight.

2.5 Germination Assessment

Ten seeds were surface sterilized with 0.1 % $HgCl_2$ solution for 15 min and then treated with 0.01 % dimecron for 10 min to prevent fungal infestation. These seeds were then thoroughly washed 5–6 times with glass distilled water and then placed in Petri dishes containing 25 ml distilled water, for germination, in dark at 32–34

°C. Germination was scored by emergence of radicle (5–7 mm in length) and expressed as percentage of seeds germinated in each Petri dish. Five such replicates were used for germination studies.

2.6 Total Protein – Extraction and Estimation

Total proteins were extracted by following the method given by Farrant et al. (1992). Five grams of the frozen sample (embryonic axes or cotyledons) was homogenized in 5 ml extraction buffer (50 mM tris-HCl, 0.7 M sucrose, 50 mM EDTA, 0.1 M KCl, 0.1 ml 2 % mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 10 mM thiourea and 2 % polyvinyl pyrrolidone. The mixture was chilled and 5 ml of water saturated phenol was added to it and shaken rigorously. The mixture was centrifuged at 8000 g for 10 min. The supernatant was collected and mixed with 5 volumes of 0.1 M ammonium acetate (methanolic). The mixture was incubated at –5 °C overnight. The pellet was obtained by centrifuging at 8000 g for 15 min and was repeatedly washed with 0.1 M ammonium acetate (methanolic). The pellet was finally washed with chilled acetone and centrifuged at 9000 g for 5 min. The proteins were suspended in 0.1 N NaOH and used for analysis of total proteins. Protein was estimated spectrophotometrically (ATI Unicam, UV2) following the method of Lowry et al. (1951) and expressed as mg protein/g fwt of tissue.

2.7 Protease Activity

The acetone purified enzyme proteins were isolated as described elsewhere (Naithani 1987). The purified enzyme was suspended in phosphate buffer (0.02 M, pH 7) and used as a source of enzyme. The assay was carried out by incubating 1 ml of the enzyme extract with 1 ml of 1 % casein and 1 ml of 0.02 M phosphate buffer of pH 7. The mixture was incubated at 37 °C for 3 h after which the reaction was terminated by the addition of 1 ml of 10 % TCA. The precipitated material was removed by centrifugation at 8000 g for 10 min and then cleared supernatant used for the determination of amino nitrogen as described by Yemm and Cocking (1955). Protease activity was recorded at 570 nm and expressed as A_{570}/min/mg protein.

3 Results

3.1 Desiccation and Loss of Viability

Sal seeds exhibited gradual loss of moisture content from 42.1 to 19.5 % within 8 dah during storage (Table 1). Absolute germinability was maintained only up to 3 dah. Desiccation of seeds to 26.5 % moisture content on 4 dah and below lead to rapid loss of viability. The seeds become non-viable within 6 dah. Seeds from 0 dah to 3 dah showed 100 % germination within 40–50 h but later on (from 4 dah) displayed delayed germination with advance in age. Strong positive correlations ($R^2 = 0.99$, $P = 0.01$; $y = –0.0006x^5 + 0.0988x^4 – 6.275x^3 + 195.48x^2 – 2972x + 17602$), which is a characteristic feature of desiccation sensitive seeds, exist between seed moisture and viability.

Table 1. Decline in germination and seed moisture content (fresh weight basis) with ageing.

Days after harvest (dah)	Seed moisture content % (f wt basis)	Germination %
0	42.1	100
0.5	41.4	100
1	40.6	100
1.5	39.8	100
2	39.3	100
2.5	37.5	100
3	36.7	100
3.5	33.9	88
4	26.5	67
5	24.6	30
6	21.8	0
7	20.1	0
8	19.5	0
9	17.5	0
10	15.8	0

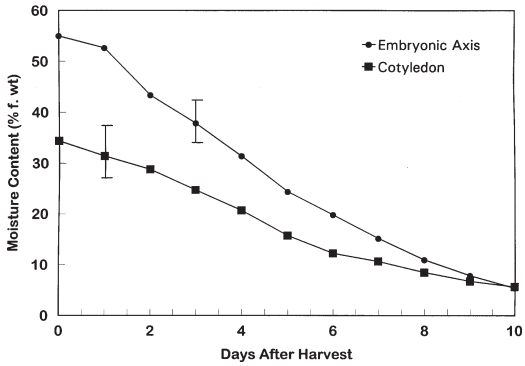

Fig. 1. Change in moisture content in both embryonic axes and cotyledons of sal seed during storage. Vertical bars represent maximum ± SD.

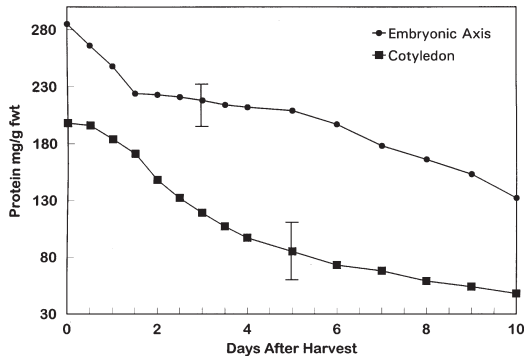

Fig. 2. Total protein content in the embryonic axes and cotyledons of sal seeds during ageing. Vertical bars represent maximum ± SD.

3.2 Moisture Content: Embryonic Axis and Cotyledon

Like seed, the moisture content of the embryonic axes and cotyledons also declined gradually with desiccation. Although, the magnitude of loss of moisture content of axis and cotyledon was similar, they differed significantly in their initial moisture content (Fig. 1). The moisture content of embryonic axis and cotyledon of freshly harvested seeds (0 dah) was 54.9 and 34.3 % respectively (Fig. 1). Highly significant positive relationship was established between seed viability and moisture content of embryonic axis ($R^2 = 0.996$, $P = 0.01$; $y = 5E-06x^5 - 0.0008x^4 + 0.0347x^3 - 0.6499x^2 + 10.197x - 116.72$) and cotyledon ($R^2 = 0.996$, $P = 0.01$; $y = 0.0021x^4 - 0.2011x^3 + 6.7113x^2 - 85.221x + 361.76$).

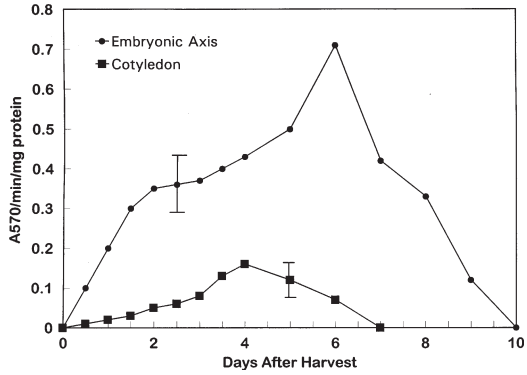

Fig. 3. Changes in the protease activity in the embryonic axes and cotyledons of sal seed during storage. Vertical bars represent maximum ± SD.

3.3 Total Protein: Embryonic Axis and Cotyledon

Gradual loss of total protein content was observed in embryonic axis and cotyledon (Fig. 2). In the embryonic axis, the protein content decreased rapidly from 285 ± 0.16 mg/g fwt (0 dah) to 224 ± 0.18 mg/g fwt (1.5 dah). Later on, the decline in protein content was relatively slow. Compared to embryonic axis, the cotyledon showed lesser amount of total protein content. Highest protein content (198 ± 0.11 mg/g fwt) recorded in cotyledons of 0 dah seed declined gradually to 48 ± 0.04 mg/g fwt on 10 dah, in non-viable seed. Highly significant correlation of total protein content in embryonic axis ($R^2 = 0.983$, $P = 0.01$; $y = 0.0001x^5 - 0.0172x^4 + 0.8394x^3 - 20.475x^2 + 258.86x - 1220.8$) and cotyledon ($R^2 = 0.992$, $P = 0.01$; $y = -2E-05x^6 + 0.004x^5 - 0.2729x^4 + 9.683x^3 - 187.63x^2 + 1887.6x - 7675.6$) was established with desiccation.

3.4 Protease: Embryonic Axis and Cotyledon

Initially no protease activity was detected in the 100 % viable embryos of 0 dah seed. Within 12 h of storage, measurable protease activity was discernible in both the tissues of embryo (Fig. 3). The protease activity increased relatively rapid with higher rates in embryonic axis than in the cotyledon and reached maximum on 6 dah (0.71 ± 0.04 / min/mg protein) and 3.5 dah (0.16 ± 0.01 / min/mg protein) in axis and cotyledon respectively. Later on, protease activity decreased sharply in both the tissues (Fig. 3). The protease activity was not detected in embryonic axis and cotyledon of 10 and 7 dah seed respectively.

4 Discussion

The desiccation-sensitive sal seeds exhibited rapid rates of loss of viability when they undergo desiccation naturally, during storage, below a relatively high moisture content (36.7 %), thus, establishing its true recalcitrant nature and confirming our previous findings (Chaitanya and Naithani 1994). Rapid rates of dehydration were also observed in the seed components, embryonic axes and cotyledons, although the hydration levels were significantly lower in cotyledons (Fig. 1). The chemical composition of the cotyledon, being rich in oils (Bringi 1987), appears to be responsible for difference in initial moisture content as reported in recalcitrant *Quercus rubra* (Pritchard 1991) and *Q. rubur* (Finch-Savage 1992).

The sal seeds exhibited gradual loss of total protein during storage (Fig. 2). Declining amounts of protein, estimated in embryonic axis and cotyledon (Fig. 2), were closely related with the rate of dehydration of these tissues and not with the germinability of seed *per se*. Dehydration of seeds from 42.1 to 36.7 % moisture content (LSMC) lead to corresponding reduction of moisture contents and total protein contents in axes and cotyledons, when the seed viability was still 100 %. For example, compared to freshly harvested seeds (0 dah) the decline of moisture content in axes (by 34.5 %) and cotyledons (by 33.4 %) of 3 dah seeds (100 % viable) resulted in concomitant loss of total protein by 43.7 and 52.6 % (of total loss) respectively. It clearly indicates that, the dehydration of seeds during storage, perhaps leads to loss of seed vigour due to rapid decline in protein content and subsequently to loss of viability. Massive loss of cellular protein was shown in ageing rice (Prabhakar and Mukherjee 1980), sal (Nautiyal and Purohit 1985), *Pisum sativum*, *Phaseolus aureus* and *Citrus reticulata* (Samshery and Banerjee 1979). Aged seeds with lower protein content, often exhibit reduction in vigour (Byrd and Delouche 1971) and are unable to withstand desiccation (Stewart and Bewley 1981).

Relatively higher levels of protein in embryonic axes compared to cotyledons of sal seeds at all stages of ageing is in close agreement with the observation of Gill et al. (1981) in *Abelmoschus esculentus* seeds. This difference in protein content may be due to the marked difference in the desiccation states of these tissues which in turn leads to either decreased synthesis or degradation due to enhanced proteolytic activity.

Seeds undergoing ageing, naturally or artificially (accelerated ageing), has been shown to have enhanced protease activity in *Vigna radiata*, *Cicer arietinum* (Agarwal and Kharlukhi 1987) The enzyme activity declined substantially in non-viable seeds of *Citrus reticulata* and *C. volkamalarin* (Samshery and Banerjee 1979). Similar increase in protease activity was recorded in embryonic axes and cotyledons of ageing sal seeds up to 6 and 4 dah respectively (Fig. 5). The enhanced protease activity in both the parts of the embryo, embryonic axis and cotyledon, of ageing sal seeds may account for the concomitant loss of total protein content in these tissues. However, protease activity alone did not seem to be responsible for decreased levels of total protein, because relatively higher rates of increasing protease activity in embryonic axes and lower rates in cotyledon did not results in corresponding loss of protein content in these tissues. Alternatively, the protein synthesizing capacity may be detrimental for the differential rate of protein loss in embryonic axes and cotyledons as relatively faster rates of desiccation in cotyledons may damage the protein synthesizing system resulting in reduced protein level. Differential rates

of protein synthesis in embryonic axis and endosperm has been suggested in *lettuce* (Black and Richardson 1967).

Substantially higher amounts of protease activity in embryonic axes than in the cotyledons might underline a special role played by embryonic axes in inducing protease activity in storage tissues. Higher protease activity was reported in embryos of quiscent seeds (Osborne 1980). The axes seem to be essential for developing protease activity in cotyledons of *Macrotyloma uniflorum* (Karunagaran and Rao 1990), pea (Varner et al. 1963, Yomo and Varner 1973) and *Cucurbita maxima* (Penner and Ashton 1967) seeds. The embryonic axes of these seeds produce a hormone, probably cytokinin (Gepstein and Ilani 1980), which promotes development of protease activity in the cotyledon (Penner and Ashton 1967). The increased protease activity may be due to *de novo* synthesis although the rate at which new enzymes appear declined in deteriorated seed, possibly due to reduced *de novo* protein synthesis (Osmond et al. 1975).

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Total of 37 references