Ascorbic acid metabolism in ageing recalcitrant sal
(Shorea robusta Gaertn. f.) seeds

K S Krishna Chaitanya, Ranjana Naithani & S C Naithani*
Seed Biology Lab, School of Life Sciences, Pt. Ravishankar Shukla University, Raipur 492 010, India
Received 24 April 1999; revised 23 May 2000

Changes in ascorbate content and its enzymatic utilization pattern were studied in embryonic axes and cotyledons of sal seeds undergoing rapid loss of viability, at ambient conditions. Ascorbate levels were significantly higher initially in the embryonic axes (0.32 mg/g fresh weight) and cotyledons (0.21 mg/g fresh weight) of freshly mature, relatively hydrated (42.2% moisture content) and 100% viable sal seeds. It declined sharply as the tissues; embryonic axes and cotyledons, desiccated with absolutely no detectable amount in non-viable seeds (21% moisture content). Significantly strong correlation was obtained between desiccation of embryonic axes (r = 0.96) and cotyledon (r = 0.97) with loss of ascorbate levels and loss of germinability. Higher rates of ascorbic acid utilization (AAU) recorded in the embryonic axes of 100% viable seed declined sharply as the seed viability reduced due to desiccation below 36.8 % moisture content. AAU was not detected in the cotyledons.

Desiccation of seeds during storage has frequently been related to oxidative injury. Free radicals of O₂ and its derivatives have been suggested to play an important role in declining seed viability, mostly by lipid peroxidation, protein and nucleic acid damage. Plants have developed complex antioxidant system[s] to protect cellular membranes from the damaging effects of 'O₂' radicals. Antioxidants like glutathione, alpha tocopherol and ascorbate are considered as chain breaking antioxidants in the free radicals mediated peroxidation due to their free radical scavenging abilities. Ascorbic acid plays a key role in detoxification of 'O₂' radicals. It can react directly by reducing superoxide, hydrogen peroxide, and hydroxyl radical, or quenching singlet oxygen. Alternatively, it can react indirectly by regenerating alpha tocopherol from alpha chromanoxy radical.

The loss of ascorbate pool resulting from desiccation alone is of particular importance and failure to maintain it is an indicator of overall degeneration in the capacity to withstand oxidative conditions. Desiccation remarkably reduces ascorbate levels in developing and hydrated seeds. Further, the ascorbate contents, which were similar in desiccation tolerant and sensitive bryophytes reduced as a result of desiccation. Ascorbic acid is known to be acted upon by many enzymes viz., peroxidase, polyphenol oxidase, and specific ascorbic acid oxidase. The net results of the activities of these enzymes is termed as ascorbic acid utilization. Higher levels of free ascorbic acid and AAU, leading to higher turnover, have often been correlated with higher growth potential during seed germination.

Our previous work showed that the sal seeds are desiccation-sensitive and deteriorate rapidly due to oxidative stress. Massive membrane perturbation caused primarily due to enhanced oxygen free radicals mediated lipid peroxidation has been suggested to be the major event during desiccation induced loss of viability in these seeds.

The present experiments have been carried out on the ascorbic acid content and its utilization in sal seeds undergoing rapid dehydration as a function of ageing, with the intent to examine the role of ascorbic acid and its metabolism during loss of viability.

Materials and Methods

Collection of seed — Physiologically mature sal seeds, 63 days after anthesis, were collected during 3rd week of May from Gariyabandh Forest (90 km from Raipur) for this study. The Gariyabandh Forest Reserve is situated to the northeast of Raipur and lies between 20°38'N latitude and 82°04' E longitude. Nearly 25-30 elite trees in the forest were marked for collection of fruits and seeds. Seeds were manually plucked and fresh mature seeds (showing 100% germination within 40-48hr) collected were brought to the laboratory within 4-5hr. The calyces of the

* Correspondent author : Email : naithani@mantraonline.com
seeds were plucked manually and healthy uninfected seeds of uniform size were sorted out and stored in perforated trays at ambient conditions [40-45% RH and 27-32°C] and used at desired intervals for the analyses.

**Moisture content** — The moisture content of the seeds, embryonic axes and cotyledons were estimated as percentage water of fresh weight. Three replicates of 15 seeds each of axes and cotyledons were weighed before and after drying at 80°C for 72hr. The moisture content was determined following the method given by International Seed Testing Association.

**Germination assessment** — Seeds were surface sterilized with 0.1% HgCl₂ solution for 15 min and then treated with 0.01% dimescon for 10min to prevent fungal infestation. These seeds were then thoroughly washed 5-6 times with glass distilled water and then placed in Petri dishes containing 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 replicates of 10 seeds each were used for germination studies.

**Ascorbic acid content and ascorbic acid utilization** — The levels of ascorbate and its utilization were measured following the method given by Chinoy et al.¹⁸ The ascorbate was extracted by homogenizing 500 mg tissue (embryonic axes and cotyledon) in a chilled mortar-pestle with 2 ml chilled CO₂ saturated water. The homogenate was transferred to a clean test tube on an ice bath. Two reaction sets were run simultaneously for determining the ascorbate content and its utilization. For measuring ascorbate, 1.5 ml of the homogenate was added to 1.5 ml buffered H₃PO₄ solution and 1 ml from this mixture was mixed with 2.5 ml of the dye and the absorbance was recorded at 540 nm. A pinch of ascorbic acid was added to the mixture to decolorize the solution and absorbance was reread at 540 nm to determine the turbidity. Ascorbate content was estimated by the difference in the two absorbances.

To determine ascorbic acid utilization (AAU), 1.5 ml of homogenate was added to 1.5 ml ascorbic acid solution and incubated at room temperature in dark for 30 min. To 1ml of the incubated mixture, 1ml buffered H₃PO₄ was added to stop the reaction. 1ml of the reaction mixture was pipetted out and 2.5 ml dye was added. The absorbance was recorded at 540 nm. A pinch of ascorbic acid was added to the solution and the absorbance was again taken at 540nm. The change in absorbance gave the utilization of ascorbate added. Ascorbic acid content was expressed as mg/g fresh weight sample and ascorbate utilization as mg ascorbic acid utilized/min/g fresh weight.

**Results**

**Loss of viability** — The freshly mature sal seeds during storage showed a rapid loss of viability within 6 day of storage at ambient conditions (Fig. 1). Decline in percentage germination of the seeds was from 100% initially to 70% on 4 day and 30% on 5 day to 0% by 6day. A gradual decrease in the percentage moisture content of the seeds was also discernible. The moisture content on harvest was 42.2%. The lowest safe moisture content was recorded to be 36.7% on 3day (Fig.1). The moisture content decreased sharply to 21% by 6day when no seeds showed germination. The fresh seeds showed germination within 40 - 48hr whereas, showed delayed germination with ageing after 3 days.

The moisture content as well as the rate of desiccation in the specific tissues, viz., embryonic axis and cotyledon varied during storage (Fig. 2). The embryonic axes of 100% viable seeds recorded higher moisture content (54.9%) as compared to cotyledon (34.4%) from mature freshly harvested sal seeds. The moisture content of the embryonic axes declined sharply to 43.3% on 2 day and finally to 19.8% in the non-viable seeds on 6 day. Desiccation of cotyledons exhibited a trend similar to the desiccation of embryonic axes. The cotyledons with comparatively lower moisture registered rapid decline in moisture

---

![Fig. 1—Decline in percentage germination (●) and moisture content (×) with age of mature sal seeds during storage. Each value is a mean of 50 observations. Vertical bars represent mean ± SD.](image)
content from 34.4% on 0 day to 24.7% on 3 day and eventually to as low as 12.2% on 6 day of storage. The rate of desiccation was faster in embryonic axes than the cotyledon.

**Ascorbic acid content** — The total ascorbate content reduced sharply in both the embryonic axes (Fig. 3) and cotyledons (Fig. 4) with the decline in moisture content during storage. The ascorbic acid content in the embryonic axes of 100% viable seeds was highest (0.32 mg/g fresh weight) and decreased gradually with the reduction in moisture content (Fig. 3). Later, it reduced gradually to 0.18 mg/g fresh weight at 36.6% moisture content and the lowest 0.12 mg/g fresh weight in the embryonic axes of non-viable seeds with 21.8% moisture content. No measurable ascorbate content was recorded thereafter.

A strong positive correlation was established between the decline in ascorbate levels and loss of viability \( r = 0.96, P < 0.001 \). Similarly, a declining trend in the ascorbate content in the cotyledon of deteriorating sal seeds was discernible, though lower levels of the same were observed in the cotyledonary tissues (Fig. 4) than the embryonic axes. With the fall in moisture content, there was a rapid loss in the ascorbate content in the cotyledon from 0.21 mg/g fresh weight in 100% viable seeds (42.2% moisture content) to 0.11 mg/g fresh weight in seeds with 39.3% moisture and no measurable ascorbic acid was found in the cotyledons with moisture content as high as 36.7% (3 day). A strong positive correlation was observed between the decline in ascorbic acid content and percentage germination \( r = 0.97, P < 0.001 \).

**Ascorbic acid utilization** — The utilization of ascorbic acid by the seed tissues was also measured (Fig. 5) though no measurable AAU was recorded in the cotyledonary tissues. AAU was registered to be 0.53 mg ascorbate utilized/min/g fresh weight in the embryonic axes of 0 day seeds with 42.2% moisture content. In the initial stages of desiccation, the axial tissues exhibited higher utilization of ascorbate, which later on declined sharply. These levels gradually increased to 0.76 mg ascorbate utilized/min/g fresh weight with initial desiccation of the seeds to 36.7% moisture content and thereafter, a steep decline in AAU levels was discernible in seeds.

![Fig. 3](image3.png) — Relationship between moisture content and ascorbic acid content in the embryonic axis of naturally ageing sal seeds. *Inset*: showing the positive correlation between loss of viability and ascorbate content in the embryonic axis \( r = 0.96, P < 0.001 \). Vertical bars represent mean ± SD.

![Fig. 4](image4.png) — Decline in ascorbic acid content in the cotyledon of sal seeds with the relative loss in moisture content. *Inset*: showing the positive correlation between loss of viability and ascorbate content in the cotyledon \( r = 0.97, P < 0.001 \). Vertical bars represent mean ± SD.

Fig. 2 — Decline in moisture content with age in embryonic axis (⦁) and cotyledon (⦁) of sal seeds during storage. Vertical bars represent mean ± SD.
with low moisture. AAU was not detected in non-viable seeds.

**Discussion**

The mature sal seeds exhibited viability up to 6 days, shortest period so far known, during storage at ambient conditions. Desiccation of seeds below 36.8% leads to rapid loss of viability (Fig. 1). These results confirm our previous findings that the sal seeds being highly desiccation sensitive lose viability very rapidly and are classified as true recalcitrant. Significantly higher rates of desiccation were observed in the embryonic axes compared to cotyledon with relatively lower initial moisture content (Fig. 2). The chemical composition of the cotyledon, being rich in oils, appears to be responsible for lower initial moisture content as also reported in *Quercus rubra* 20.

Sharp decline in ascorbate levels recorded in desiccating embryonic axes and cotyledons of sal seeds was similar to the results obtained for other seeds 21 and vegetative tissues 22. Presence of higher amounts of ascorbate in freshly harvested sal seeds can be attributed to the unique developmental feature of recalcitrant seeds including seeds of *Shorea robusta* (Figs 3 and 4). These seeds do not undergo maturation drying during development and therefore, they are shed with relatively high moisture content 23. Higher amounts of ascorbate have been shown to be a characteristic feature of developing *Vicia faba* seeds (with maximum moisture content), and their levels decline concomitantly with the reduction in moisture content during maturation 24. Further, relatively higher amounts of ascorbate may effectively suppress the desiccation induced oxidative stress leading to loss of viability in sal seeds. Remarkably lower levels of ‘O₂⁻’ radical and its mediated lipid peroxidation in 100% viable seeds could possibly be due to antioxidant ability of ascorbate to inhibit the lipid peroxidation or directly scavenging ‘O₂⁻’ radicals. Significantly strong correlation between desiccation of embryonic axes (r = 0.96, P<0.001) (Fig. 3 - inset) and cotyledons (r = 0.97, P<0.001) (Fig. 4 - inset) with loss of ascorbate further establishes the desiccation sensitivity of sal seeds. Comparatively higher amounts of ascorbate along with lower rates of its decline in desiccating embryonic axes than in the cotyledons of ageing sal seeds, appears to be due to differential level of desiccation of these tissues.

Degradation of ascorbate during desiccation has been found to be a common phenomenon in several plant species 22-24 and may be ascribed to a corresponding rise in the ascorbate oxidase activity 25. In sal seeds, the ascorbate utilization is detected only in the embryonic axes and not in the cotyledons. The embryonic axes of 100% viable seeds showed higher rates of ascorbic acid utilization and as the seed deteriorates (i.e., after 3 days) a sharp loss in ascorbic acid utilization was also discernible, with no ascorbic acid utilization in non-viable seeds (Fig. 5). The data of Shigeoka et al. 26, Tommasi et al. 27 and Arrigoni et al. 28 suggested that decrease in ascorbate and ascorbate utilization occurring during ageing can be correlated with the onset of biochemical pathway leading to loss of viability in seed 28.

**Acknowledgment**

The authors acknowledge Prof. M L Naik, Head, School of Life Sciences, Pt. Ravishankar Shukla University, Raipur for providing necessary laboratory facilities. Financial assistance to KSK by Madhya Pradesh Council of Science & Technology, Bhopal (B-47990) is also acknowledged.

**References**


15 Naithani, S C, In Hormonal regulation of developing cotton hair, Ph D Thesis (Saurashtra University, Rajkot, India) (1983) 71.


