Characterization of seeds of selected wild species of rice (Oryza) stored under high temperature and humidity conditions

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Wild progenitors of rice (Oryza) are an invaluable resource for restoring genetic diversity and incorporating useful traits back into cultivars. Studies were conducted to characterize the biochemical changes, including SDS-PAGE banding pattern of storage proteins in seeds of six wild species (Oryza alta, O. grandiglumis, O. meridionalis, O. nivara, O. officinalis and O. rhizomatis) of rice stored under high temperature (45°C) and humidity (~100%) for 15 days, which facilitated accelerated deterioration. Under the treated conditions, seeds of different wild rice species showed decrease in per cent germination and concentrations of protein and starch, but increase in conductivity of leachate and content of sugar. The SDS-PAGE analysis of seed proteins showed that not only the total number of bands, but also their intensity in terms of thickness differed for each species under storage. The total number of bands ranged from 11 to 22, but none of the species showed all the bands. Similarity index for protein bands between the control and treated seeds was observed to be least in O. rhizomatis and O. alta, while the indices were 0.7 and 0.625 for O. officinalis and O. nivara, respectively. This study clearly showed that seed deterioration led to distinctive biochemical changes, including the presence or absence as well as altered levels of intensity of proteins. Hence, SDS-PAGE protein banding pattern can be used effectively to characterize deterioration of seeds of different wild species of rice.

Keywords: Oryza, Rice, Wild species, Paddy seeds, Biochemical changes, Proteins, SDS-PAGE

Wild species of crops which include crop ancestors as well as relatives have been an important part of crop improvement programmes. Genes from wild species have provided crops with resistance to pests and diseases, enhanced tolerance to abiotic stresses, improved quality traits such as protein content, and have made possible the production of hybrid varieties. Genus Oryza has 23 species, of which 2 are cultivated (O. sativa and O. glaberrima) and others are wild species, which also carry many useful genes for rice improvement. Oryza meridionalis and O. rhizomatis have drought avoidance genes, while O. grandiglumis and O. alta have high biomass production potential. Similarly, O. nivara possess grassy stunt virus resistance gene while O. officinalis has resistance to many diseases.

Storage properties of rice seeds2, particularly in case of wild species influence the frequency of germplasm regeneration, the amount of seed required for production, seedling vigor, and the risks of seed production and management, especially under wet weather conditions in the tropical regions. Wild rice seed germination is often intolerant of temperatures in excess of 40°C. Genetic research on seed storability in rice has also indicated that there are significant differences in storage properties among germplasm accessions from different geographical regions3,4. Generally, the seed storability using the storage property is measured by the difference of seed germination rates before and after treatment of rice seeds under 40°C and 95% relative humidity for 10 days5.

The major storage protein in rice which accounts for about 70-80% of total proteins is glutelins6. Protein electrophoretic techniques have been used for varietal identification of paddy (seeds)7. Studies on the banding pattern of soluble protein of indica, japonica and javanica rice ecotypes have revealed that their protein banding patterns could easily differentiate these ecotypes8. The electrophoretically detectable protein polymorphism in rice grain is also used to indicate geographic origin as well as breeding improvement level9.
In the present study, the biochemical changes, including the SDS-PAGE banding pattern of storage proteins have been characterized in seeds of six wild species of rice, stored under accelerated deteriorating conditions.

Materials and Methods

Freshly harvested seeds (paddy) of six wild species of rice (*Oryza officinalis*, *O. nivara*, *O. grandiglumis*, *O. meridionalis*, *O. rhizomatis* and *O. alta*) were obtained from the experimental *Oryza* garden of Central Rice Research Institute, Cuttack, Orissa. The seeds were screened with the magnifying lens and only the intact seeds without visible defects, insect damage or malformations were selected. Since wild rice seeds exhibit dormancy, seed dormancy was broken by the standard method\(^{10}\).

Replicated samples of harvested seeds were stored under extreme conditions of high temperature (45°C) and moisture (100% relative humidity) for 15 days. These were taken as treated seeds and untreated seeds were considered as control. Moisture content was determined in three replicates by oven drying the seeds (100 nos.) at 95°C for constant weight\(^{11}\). Before analysis, the moisture content of the treated and control seeds were adjusted to be the same by drying them at 35°C.

For the seed leakage test, three weighed replicates of seeds (2 g) from each treatment were soaked in 50 ml of distilled water at 25°C for 16 h. A control with distilled water (but without seeds) was also maintained. The leachate from seeds was collected and the electrical conductance (EC) was measured with a digital conductivity meter.

Germination test was conducted using the seed germination papers in four replicates of 50 seeds each at 25°C\(^{10}\). The germinated seeds were evaluated into different categories as normal and abnormal seedlings and the dead and hard seeds were counted on the 14\(^{th}\) day. The germination percentage was recorded on the basis of normal seedlings only. For estimation of protein content, seed sample (0.5 g) was mashed with 5 ml of 0.05 M tris HCl buffer (pH 7.2) and transferred to the centrifuge tube. The extract was then centrifuged at 1200 \(\times\) g for 15 min at 25°C and decanted into a 50 ml beaker. This extraction was repeated three more times. The alcohol extract was evaporated on a water bath at 80-85°C until most of the alcohol was removed (e.g. volume was reduced to about 3 ml) and made up to 25 ml with distilled water. Sugars in this sugar extract were analyzed\(^{13}\) and the amount of sugars present in the extract was determined using a standard curve prepared from glucose. Starch content was estimated in the residue left after sugar estimation\(^{13}\).

For total protein extraction, kernels (1 g) of each species were crushed between folded papers with a hammer on a metal plate. From the crushed kernels, a sample (500 mg) was transferred into an eppendorf tube and sample buffer containing 2% \(\beta\)-mercaptoethanol (2 ml, pH 6.8) was added to it. Extraction was done for at least 1 h at 37°C in incubator. Then it was centrifuged for 10 min at 12,000 \(\times\) g just before loading the supernatant in the gel slab (with 13 well comb and 1.5 mm thick gel).

Seed proteins were characterized by the SDS-PAGE\(^{14}\), with some modifications using vertical gel electrophoresis system (Genei, Bangalore, India). Samples were run in denaturing gel system with 4% stacking gel and 10% separating gel at 150 volts. Protein samples (20 µl each) containing 20 µg of protein were loaded and run till the dye reached the bottom. Staining was done through Coomassie brilliant blue R-250 and destaining through 5% NaCl solution.

For analysis of banding pattern, the total number of bands were counted and further classified into following groups according to their thickness and intensity: thick, - relatively thick as compared to other protein bands; medium, -in between thick and thin protein bands; thin, - relatively thin as compared to other protein bands, and faint, -slight appearance of protein bands. In addition, the densitometric values were considered for this analysis.

The gel was scanned in Flurochem\(^{TM}\) 5500 (Alpha Innotech, California, USA) Densitometer equipped with UVI Tech software (St. John’s Innovation Center, Cambridge, UK). Similarity coefficient = No. of same bands/(No. of same bands + No. of different bands) was used for calculating the similarity between control and treated seeds. Both the control and treated were considered similar, if the similarity coefficient value was 1 and different if it was 0.
Results
The seeds of wild species of rice showed significant differences in various biochemical parameters tested after storage under high temperature (45°C) and humidity (~100%) for 15 days. In general, treated seeds had lesser per cent germination (Fig. 1), increased seed leachate conductivity (Fig. 2), decreased protein content (Fig. 3), increased sugar content (Fig. 4), and decreased starch content (Fig. 5). Significant minimum decreases in protein (21.23%), starch (14.11%) and per cent germination (13.14%) as well as minimum increases in sugar content (16.36%) and seed leachate conductivity (17.16%) were observed in *O. officinalis*. However, species like *O. alta* showed maximum changes in all the biochemical parameters tested. In general, treated (aged) seeds had higher solute leakage than control seeds of respective wild species (Fig. 2).

In the electrophoretic study, specific protein band patterns were obtained which were characteristic of each wild species under a particular storage condition (control or treated) (Fig. 6). The results of SDS-
PAGE gel analysis of seed proteins obtained for six wild rice species under control (fresh) and treated (aged) conditions are presented in Table 1 and 2. The results summarized in Table 1 revealed that the wild species differed from each other with respect to the total number of bands, and also in the number, thickness and intensity of each protein band, more so in the case of treated seeds.

The total number of bands in the SDS-PAGE gels ranged from 11 to 22. A maximum of 22 bands were observed in control (fresh) seeds of *O. grandiglumis*. The thickness and intensity of various bands differed between the control and treated seeds as well as among the different wild species (Figs 7 & 8). This provided additional means of distinguishing the control and treated seeds of the same species. On the basis of thickness, protein bands were categorized into four groups namely thick, medium, thin and faint and their total numbers in each group varied from 2 to 6, 1 to 5, 1 to 5, 3 to 10 respectively. Under control conditions, *O. grandiglumis* had the maximum number of thin bands. The seeds of *O. nivara* and *O. rhizomatis* had only one medium thick band under treated conditions. In general, control seeds had more number of thick bands than treated seeds. All the selected wild species showed varied number of faint bands under control conditions, more so under treated conditions. When the total proteins of the aged seeds were electrophoretically analyzed on SDS-PAGE (Fig. 7), it was found that the highest molecular subunits (more than 66 kD) were disintegrated into low molecular mass polypeptides (below 43 kD). This was clearly evident in the densitograms (Fig. 7), as the intensity of the high molecular weight subunits decreased with the loss of viability of the seeds in the aged samples.
Based on this densitogram, a zymogram was drawn showing the thickness and intensity of band (Fig. 8). From the zymogram the total number of bands and those in each group were counted and presented in Table 1. The similarity coefficients between control and treated pairs of wild species were calculated based on relative mobility of protein bands and presented in Table 2. A close perusal of Tables 1 and 2 revealed that variations existed among different wild species in general and between the control and treated seeds of the same wild species, in particular with regard to the polypeptide bands. This provided adequate basis to distinguish them from each other.

The homology of control and treated seeds (similarity coefficient) for protein bands varied from 0.70 to 0.2857 in different species of rice tested. Results on similarity coefficient (Table 2) revealed that O. nivara (0.7) and O. officinalis (0.625) showed maximum similarity between treated and control seeds. On the other hand, O. alta (0.2857) and O. rhizomatis (0.3157) showed minimum similarity between control and treated seeds. Thus, it was observed that seeds of particular species showed typical variation between control (fresh) and treated (aged) storage conditions.

**Discussion**

Utilization of genetic resources in crop species, particularly those in the gene pool of wild species may provide further opportunities for crop improvement. Through millions of years of evolution and genetic adaptation to environments, the wild species have accumulated abundant genetic diversity, which is beneficial to the improvement of cultivated species. In the present study, seeds of wild species of rice, namely O. alta, O. grandiglumis, O. meridionalis, O. nivara, O. officinalis and O. rhizomatis were characterized for changes in biochemical parameters when stored under high temperature and humidity conditions. As better understanding on seed viability of these species may be useful for seed testing, seeds under control (fresh) and treated (aged) conditions were used. The seeds of selected wild species showed significant changes in the biochemical parameters, especially the SDS-PAGE protein banding pattern under control and treated conditions.

The loss in seed viability and protein content are due to the fact that cellular membranes are composed primarily of proteins and lipids. During the seed deterioration process, disorganization of proteins and lipid phase transitions influence the membrane structure and integrity, consequently seed viability. Likewise, the changes in the conductivity of seed leachate during seed deterioration are often attributed
to the membrane reorganization. The tonoplast and plasma lemma, which normally retain solutes within cells, lose their integrity during seed deterioration and do not act as retentive barriers when seeds are first placed in water\(^\text{17}\).

The inability of seeds to germinate can be related to the difficulties in the membrane competency. But, in the present study, the seeds were experimentally subjected to high temperature and humidity conditions to obtain non-germinating seeds. The use of these seeds helps to gain greater insights on the seed deterioration mechanism. Higher leachate conductivity in non-germinating seeds could be due to the enhanced seed membrane permeability. The pattern of changes in electrolytic leakage is often used to estimate the damage to cell membranes in several other crop seeds\(^{18,18}\).

As seeds age, membranes become leaky, enzymes lose catalytic activity, seed food reserves deplete and the byproducts of catabolic reactions become toxic\(^\text{19}\). Other time-dependent changes like the loss of protein content and increase in soluble sugar contents may lead to irreversible degradation of important cellular machinery\(^\text{16}\). Any increase in leachate conductivity is primarily a function of loss of the cell membrane integrity. Seed viability loss is often attributed to the loss of integrity of the plasma lemma\(^\text{16,19}\). Increased leakage of ions, amino acids and sugars from aged seeds during storage is a definitive sign of loss of permeability of the plasma membranes. The loss of germinability with ageing coincides with considerably increased plasma membrane permeability and changes in seed water status\(^\text{18}\). The increase in electrolytic leakage, a direct measure of loss of membrane integrity and the corresponding loss of viability of the threshold value, therefore, show the accumulation of injured cell above a critical level\(^\text{11}\).

The seed proteins, when denatured by heating in the presence of excess SDS and 2-mercaptoethanol bind with SDS in constant weight ratio, as if they have essentially identical charge density. This complex migrates in polyacrylamide gel of the correct porosity according to polypeptide size. In the present study, the total number of protein bands observed was less in treated than in control seeds. Among the treated seeds, \(O. \text{ alta}\) showed the least number of bands (11). The above findings indicated that different species can be distinguished on the basis of the total number of protein bands. Likewise, within each wild species, the treated and control seeds can be distinguished on the basis of the protein banding pattern.

The correlation between the similarity coefficient and loss in per cent germination was highly significant (\(r^2 = 0.932\)). The present investigation revealed that the control and treated seeds of a particular species differing in their seed viability can be differentiated on the basis of the number of protein bands at the gross level, and intensity of bands or similarity coefficient (per cent) with precision. The study clearly showed that seed deterioration was not only accompanied by decrease in per cent germination, protein and starch content, but by increase in seed leachate conductivity and sugar content in all the wild species of rice tested.

The present study showed that banding pattern in SDS-PAGE gel was found to be affected by treated (ageing) condition. Many studies\(^{7,20-22}\) have reported similar changes in number and thickness and relative mobility in protein banding pattern of seeds from different rice cultivars. The results of the present study clearly demonstrated the differences in banding pattern due to the loss of seed viability. This technique is simple, quick and economical and requires very little quantity of seed (500 mg powder). In addition, this method of seed characterization can be used for paddy seeds which are stored in small quantities (to save space), such as in many germplasm collection centers. Although there is a continual need for rapid testing seeds using non-invasive methods\(^\text{52}\), the seed protein analysis using SDS-PAGE can save time and quantity of seeds needed for conducting the germination tests.

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