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Full Length Article

Physio-biochemical and Genetic Changes in Stored Pea (*Pisum sativum*) Seeds

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Abstract

Studies were carried out to evaluate the physiological and biochemical changes in pea seeds (cv. Meteor) stored at 5±1°C and ambient temperature 25±2°C. RAPD makers were used to identify any genetic dissimilarity in pea seeds stored up to two years under different storage environments. Maximum germination (98.33%) was recorded in seeds from freshly harvest seeds and minimum was 90% in 2 years old seed stored at 5°C±1. The decline in vigor was rapid in one year seed stored under ordinary condition compared to other storage environment. The α- and β-amylase activity was higher in recent harvest seeds compared to stored seeds. The storage period showed significant effects on alpha- and beta- amylase activity and a decreasing trend was observed as the storage period prolonged. Similarly, peroxidase and catalase activity was also higher in fresh seeds in contrast to α- and β-amylase. Genetic analysis (RAPD) showed 2% dissimilarity in genetic profile amongst the seed lots. However, the seeds stored for longer period regardless the storage environment, have more genetic damage. The physiological, biochemical and genetic changes could be helpful in understanding the mechanism of pea seeds deterioration.

Keywords: *Pisum sativum*; Seed ageing; Seed storage; Germination; RAPD; POD; CAT; Amylase; DNA; Vigor

Introduction

The understanding of seed longevity has significance in germplasm conservation for future crops by establishing seed gene banks (Bewely and Black, 1994; Akhtar *et al*., 2012). Seeds being small and containing a wide range of genetic traits are the most convenient part of the plant to store. The most important aspect of germplasm conservation is maintenance of genetic integrity of seed population during storage (Breese and Taylor, 1981; Roos, 1984; Breese, 1989). Seeds are stored in gene banks under optimal storage conditions to prolong the seed viability. The deterioration is expected and usual phenomenon in stored seeds and even under ideal storage conditions they tend to lose their viability (Bhatti and Sato, 1997). During extended storage many seeds show deterioration in their germination but the tendency to deterioration differs significantly among species (Roberts, 1989). The rate of seed deterioration during storage is dependent on seed moisture, storage conditions, and crop species (Tang *et al*., 1999).

The seed possesses the highest vigor at the time of physiological maturity and gradually decreases with storage period (Goel and Sheoran, 2003). Among several variable factors, storage period (time) markedly influence the seed longevity. The climatic conditions of tropical regions accelerate the seed ageing, so the maintenance of seed viability during storage is generally a greater problem in tropical region. A major cause of low vigor has been identified due to seed ageing. Seeds show a gradual decline in vigor as they aged, leading to slower and less uniform germination. The seed ageing in both orthodox and recalcitrant species during storage deteriorates the germination of stored seeds (Murthy *et al*., 2003). Several researchers have identified different causes of seed ageing, however, among them enzyme inactivation, disruption of cellular membranes, free radical mediated lipid-peroxidation, and damage to genetic integrity are considered to be major one (McDonald, 1999).

The studies conducted to investigate biochemical deterioration during seed ageing was conducted mostly under artificial ageing (McDonald, 1999), where high temperature and humidity conditions are normally used. The seeds under such storage conditions typically lose their viability within a few weeks or sometimes even within a few days. These studies may improve our understanding of mechanisms of seed ageing; however, it is important to

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know whether the seed ageing mechanisms are same or not under accelerated and natural ageing conditions. The activities of peroxidase, catalase, ascorbate peroxidase, glutathione reductase and superoxide dismutase decreased under natural ageing conditions in cotton seeds (Goel and Sheoran, 2003).

The integrity of DNA extracted from seeds can vary depending upon seed quality or physiological state of the seed. Samples of aged seeds are known to generate more morphological variability than those from highly viable seeds. Seed ageing induces spontaneous genetic changes (point mutation or chromosome aberrations) and selection effects (genetic shift or drift). It has been reported that changes in DNA might be the primary cause of loss in viability in aged seeds (Cheah and Osborne, 1978). Recent advances in molecular biology are being utilized to better understand the mechanisms of seed deterioration in relation to DNA (Chwedozewska *et al*., 2002). Random Amplified Polymorphic DNA (RAPD) as a practical genetic identity element is useful in breeding of crop species, seed production and seed testing program. Soybean (*Glycine max*) seed samples displayed differing RAPD polymorphisms with specified primers as a response to environmental exposure using artificially aged or primed and non-primed seeds.

In view of the above we envisage that seed storage for variable intervals, storage conditions may yield variable degrees of biochemical and genetic changes that may lead to loss of seed viability or vigor. Therefore, the aim of the present study was to appraise the effects of different storage conditions on seed viability and vigor in pea seeds, biochemical profile and genetic disintegration in relation to seed ageing in response to different storage environments.

Materials and Methods

Plant Material

Seeds of pea (*Pisum sativum* L.) cv. Meteor were used to evaluate the physiological, biochemical and DNA integration during the storage. Seeds were obtained from the Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan. Seeds were randomly selected from one and two year old seed lots stored at 5^{o}C. Seeds were surface sterilized in 75% ethanol for 5 min then in 5% sodium hypochlorite solution for 10 min. The seeds were rinsed five times with sterile deionized water.

Germination Test and Seedling Growth

The standard germination test for pea was performed according to ISTA rules (ISTA, 1995). The seeds were germinated on the moist Whatman 1 filter paper in 9 cm Petri dishes. Petri dishes were placed at 25±2^{o}C under white fluorescent light, in controlled growth room. Data for germination, radicle and plumule length was recorded for the evaluation of germinability of the stored pea seeds. After seven days seedlings were transferred to pots filled with sand and peat (1:1 v/v) and were watered with Hoagland solution when needed. The established seedlings were harvested after 14 days of transplantation to measure shoot diameter, number of leaves, plant height, root-shoot fresh and dry weight, and leaf area index. Leaf area index was measured on leaf area meter.

Biochemical Assay

Extraction: Extraction for the enzymes activities was performed in potassium phosphate buffered saline (pH 7.2). Seed samples weighing (0.1 g) each of three replicates was extracted in buffer with the help of pestle and mortar; phenyl methyl sulfonyl fluoride (PMSF) (10 mM) was used as proteases inhibitor. Finally, centrifuged at 10,000 × g at 4^{o}C and supernatant was used for enzyme assay (Razzaq *et al*., 2013).

Catalase (CAT) and peroxidase (POD) activity: Activities of catalase (CAT) and peroxidase (POD) were measured following the methods of Ambreen *et al.* (2000) and Naqvi *et al.* (2011) respectively with some modifications. The CAT reaction solution (3 mL) contained 50 mM phosphate buffer (pH 7.0), 5.9 mM H_2O_2, and 0.1 mL enzyme extract. The reaction was initiated by adding the plant seed extract. Changes in absorbance of the reaction solution at 240 nm were taken after every 20 sec. One unit of CAT activity was defined as an absorbance change of 0.01 units per min. The POD reaction solution (3 mL) contained 50 mM phosphate buffer (pH 5.0), 20 mM guaiacol, 40 mM H_2O_2, and 0.1 mL plant seed extract. Changes in absorbance of the reaction solution at 470 nm were determined every 20 seconds. One unit of POD activity was defined as an absorbance change of 0.01 units per minute.

α- & β-amylase activity: Both α- & β- amylase activity of all pea seeds was assayed according to the method of Mukhtar *et al.* (2013). The activity of α-amylase was recorded at 620 nm, while that of β-amylase was made at 540 nm.

Genetic Assay

Genomic DNA extraction: Presoaked stored pea seeds (5-6 in numbers) were grounded with hot CTAB solution until complete homogenization and then the solution was transferred into zipper plastic bags and put into the water bath at 65°C for 30 min. After incubation the homogenized tissues were transferred into 1.5 mL tubes and added the chloroform isoamyl alcohol with equal volume of the homogenized solution. Centrifugation was done at 12000 × *g* for 10 min in a centrifuge (MSB010 CXI 5, MSE, UK). Approximately 700 µL of isopropanol was added into the supernatant already taken after centrifugation. DNA pellet was washed, re-suspended in 0.1 X TE buffer, and

quantified by a spectrophotometer (CECIL CE 2021 2000 Series Cambridge, UK) at 260 nm. Extracted DNA was diluted to 35 ng μL^{-1} in dH_2O to make the working dilution for PCR studies.

RAPD (PCR) analysis: Twelve highly polymorphic RAPD primers earlier reported in wheat (Khan *et al*., 2004) were selected and out of these twelve RAPD primers six primers (GLC-3, GLC- 7, GLC-8, GLC- 15, GLA- 11 and GLC- 18) showed polymorphism in this study and included in the RAPD analysis. All RAPD reactions were performed in a thermal cycler (Eppendorf, Germany) with 35 ng DNA as template per reaction. Amplification reactions contained 1.0 U of Taq polymerase (MBI, Fermentas, Vilnius, Lithuania), 3 mM $MgCl_2$, 2.5 mM of each dNTP, 15 ng of each decamer (Gene Link Company, Hawthorne, NY, USA). The reaction conditions for amplifying the genomic DNA of pea were as follows; 5 min at 94°C, then 40 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C and final extension for 10 min at 72°C. All amplification products stained with ethidium bromide were checked on 1.2% (w/v) agarose gel electrophoresis in a 1X TBE buffer at 80 V for 1 h.

Experimental Design and Data Analysis

The experiment for germination test was repeated three times with 20 seeds per replication. To quantify the growth parameters, germinated seedlings were transferred to pots and repeated three times (5 seedlings per replication). The values recorded were submitted to analysis of variance (ANOVA) under Completely Randomized Design (CRD) and means were separated by least significant difference test (LSD) using MSTAT-C statistical package (Russell, 1986). All visible and robust polymorphic RAPD fragments were counted as presence (1) or absence (0) for each sample. The data of the primers were used to evaluate the genetic similarity among the pea individual based on the number of shared amplification products.

Results

The results showed non-significant ($p>0.05$) relationship between the storage conditions and final germination percentage of pea seeds (Table 1). However, freshly harvested pea seeds showed a higher mean germination percentage. The results for shoot diameter showed a significant ($p<0.05$) relationship with storage conditions (Table 1). The seeds stored for two year at 5°C gave better results for seedling stem diameter compared to others. Significant ($p<0.01$) relationship was observed between plant height and storage conditions (Table 1). Seeds stored for two years at 5°C showed significant difference from other storage conditions for seedling height. However, it was statistically non-significant with the freshly harvested seeds. Highly significant ($p<0.01$) relationship was observed between storage conditions in relation to root and shoot fresh weight (Table 1). The LSD test for root and shoot fresh weight, confirmed that the seeds stored for two years at 5°C were significantly different from the freshly harvested seeds but were non-significant different from others. For root dry weight non-significant difference was observed between freshly harvested seeds and the seeds stored for two years at 5°C (Table 1). But they were significantly different ($p<0.05$) from others. The shoot dry weight showed a highly significant difference ($p<0.01$) between the storage conditions (Table 1). The seeds stored for two years at 5°C was significantly different from the fresh ones but was non-significantly different from others. The results for leaf area showed a significant difference ($P<0.05$) between storage conditions (Table 1). Significant difference was also observed for radicle length between different storage conditions, however, results were non-significant for the plumule length (Table 1).

The enzymatic activity in stored pea seeds showed a significant decrease with the storage time. The activity of α-amylase, β-amylase and catalase was higher in one year old seeds stored under ordinary conditions compared to one year old seeds stored at 5°C. However, peroxidase activity was higher in one year old seeds stored at 5°C than the one year old dry seeds stored under ordinary conditions. The results showed that storage time rather than the storage conditions is more important and significantly affect the enzymatic activity in pea seeds (Table 2).

Out of 12 random primers six primers showed polymorphic amplification profile and scored 33 bands (5.5 bands per primer) out of which 9 loci were found polymorphic (Fig. 1). Genetic similarity among all the samples ranged from 0.9805 to 0.9196. Maximum genetic similarity (0.9805) was estimated among the seeds stored for 2 year at 5°C (Lane B) and the seeds stored for one year under ordinary storage conditions (Lane D), whereas the genetic similarity among one year old seeds stored at 5°C (Lane C) was found to be 0.9018 with respect to other stored seed lots.

Discussion

It is known that seed deteriorate when stored for long periods. There is a loss of growth and vigor in aged seeds which ultimately cause a decrease in total number of germinating seeds. The decrease in seedling vigor depends upon the storage conditions. Storage relative humidity and temperature are the major factors, which influence the seed quality during storage. In this study pea seeds stored at different environments were evaluated. The results showed that there was a significant effect of storage conditions and period on seed vigor, enzymatic activity and DNA integration.

Previous studies on the effect of artificially aged seeds suggested considerable loss in seedling vigor with passage of ageing period (Rodo and Marcos-Filho, 2003; Khan *et al*., 2003; Abbas *et al*., 2004; Contreras and Barros, 2005; Gholami and Golpayegani, 2011). The results for

Table 1: Emergence and seedling vigor of pea seeds stored under different conditions

Storage Conditions	Germination mean (%)	Stem diameter (cm)	No. of leaves	Plant height (cm)	Root fresh weight (g)	Shoot fresh weight (g)	Root dry weight (g)	Shoot dry weight (g)	Leaf area index (cm^2)	Radicle length (cm)	Plumule length (cm)
Freshly harvested seeds	19.67± 0.33	0.23 ± 0.01a	9.73 ± 0.13	6.15 ± 0.18a	0.92 ± 0.03a	1.41 ± 0.03a	0.06 ± 0.00a	0.14 ± 0.01a	0.80 ± 0.04a	3.25± 0.06a	0.61± 0.24
Seeds Stored for 2 years at 5°C	18.00 ± 1.00	0.18 ± 0.01b	9.07 ± 0.29	6.62 ± 0.22a	0.49 ± 0.02b	0.78 ± 0.04b	0.06 ± 0.02a	0.05 ± 0.02b	0.85 ± 0.02a	3.14± 0.53a	0.96± 0.23
Seeds Stored for 1 years at 5°C	19.00 ± 0.58	0.17 ± 0.01b	8.53 ± 0.18	4.51 ± 0.52b	0.34 ± 0.03c	0.56 ± 0.05c	0.02 ± 0.00b	0.06 ± 0.01b	0.75 ± 0.01ab	1.92± 0.21b	0.82± 0.09
Seeds Stored for 1 year at ordinary conditions	18.67 ± 0.88	0.15 ± 0.02b	7.47 ± 0.93	4.01 ± 0.45b	0.26 ± 0.03c	0.47 ± 0.07c	0.02 ± 0.00b	0.04 ± 0.00b	0.62 ± 0.08b	1.80± 0.16b	1.29± 0.13
LSD	NS	0.188	NS	1.217	0.103	0.498	0.0188	0.0188	0.146	0.9656	NS

Results are the average of three determinations, ±, SD

Table 2: Profile of α, β-amylases, peroxidase and catalase in Pea seeds stored under different conditions

Storage Conditions	Enzyme Activity (U mL^{-1})			
	α-amylase	*β-amylase*	*Peroxidase*	*Catalase*
Freshly harvested seeds	4.886d	2.82def	15.48a	2.112ef
Seeds Stored for 2 years at 5°C	1.364ef	1.03ef	8.57c	0.7536ef
Seeds Stored for 1 years at 5°C	1.768ef	1.062ef	12.8b	0.5176f
Seeds Stored for 1 year at ordinary conditions	3.313de	1.6ef	10.71bc	1.262ef

Results are the average of three determinations

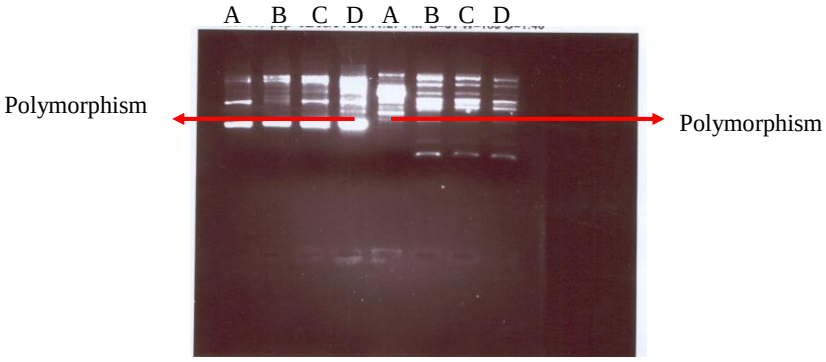

Fig. 1: Random Amplified Polymorphic DNA (RAPD) (PCR) of four pea lots stored under different conditions amplifying with Primers GLC-8 and GLA-11. Lane A is presenting freshly harvested pea seeds; lanes B, C and D are seeds stored for two year at 5°C, seeds stored for one year at 5°C and seeds stored for one year under ordinary storage conditions, respectively

accelerated-aged seeds showed significant effect on seed vigor and viability in pea (Khan *et al*., 2003). Loss of vigor, germination capacity and viability are closely associated with the process of deterioration as it affects seed quality (Trawatha *et al*., 1995). It is important to know that the pea seeds stored for one year but under two different storage conditions (5°C and under ordinary storage conditions) showed different response in relation to seedling vigor and growth. However, overall loss of seed viability was slower in freshly harvested and two year old seeds stored at 5°C compared to other seed lots. It is obvious to find that the seeds stored for two year at 5°C showed better results for seed vigor compared to those which are stored for one year on similar conditions. It is well documented that seedling vigor decreases with the storage time (Parmoon *et al*., 2013); however, our study on seed storage behavior of pea seeds showed different results. So, it is imperative to investigate for this different behavior of pea seeds during storage.

In prolonged storage, high moisture levels and temperatures cause a reduction in seed longevity and profound deteriorative biochemical changes in seed membrane (McDonald, 1999; Walters, 1998). Peroxidative changes during the process of ageing may be the major cause of seed deterioration. Protective mechanisms that could scavenge the peroxide (e.g., H_2O_2) produced as a result of stress conditions have been reported in soybean seeds (Sung, 1996). In this study it was found that in stored peas seeds α- and β- amylase activity decreases with the time of storage, regardless the storage environment. Similar reductions in the activities of various peroxide-scavenging enzymes with natural ageing in soybean seeds has been

reported (Sing and Chiu, 1995). According to Kole and Gupta (1982) a sharp decline in enzymatic activity and declined germination was observed during artificial ageing of safflower (*Carthamus tinctorius*) seeds. Bailly *et al.* (2004) stated that a tight regulation of catalase by artificial or natural drying suggests that hydrogen peroxide removal may be crucial for avoiding desiccation. In this investigation we report that decrease in peroxidase, catalase and other enzymatic activities were observed in aged seeds. Pea seeds stored for two years showed significant reduction in catalase from 2.11 U mL^{-1} (fresh seeds) to 0.75 U mL^{-1}. Peroxidase activity in aged seeds was also reduced from 15.48 U mL^{-1} to 8.57 U mL^{-1}. In their studies, Parmoon *et al.* (2013) and Demirkaya (2013) reported a significant reduction of enzymatic activities in aged milk thistle (*Silybum marianum*) and pepper seeds, respectively.

The seed may provide an ideal 'model' system through which to investigate the effects of a variety of endogenous DNA damaging agents and environmental stresses on genome integrity (Bray and West, 2005). The RAPD analysis clearly indicated the integration of DNA content in dried pea seeds from different storage regimes. One of the significant factors which may contribute to loss of seed viability during storage is the accumulation of chromosomal damage and/or an inability of the seed to repair such damage during the period of imbibition (Cheah and Osborne, 1978). Priestly (1986) reported a reduction in DNA during naturally and artificially deteriorated seeds and embryo axes of different species. Decrease in total DNA concentration of rye (*Secale cereale*) embryos was accompanied by the loss of viability (Osborne *et al*., 1981). Marcos-Filho and McDonald (1998) also reported reductions in DNA concentration in aged soybean seeds during the process of deterioration. Our investigations revealed that pea seed deterioration during storage showed some relation with DNA integration. The genetic similarity ranging from 0.9805 to 0.9196 was observed between the seed lots of same genotype, which are confirmatory to the findings of Vijay *et al*. (2009) for soybean and safflower, who used RAPD markers for this purpose.

In conclusion, this study demonstrated that there is a strong relation between seed storage environments, enzymatic and DNA changes with seed deterioration processes during storage. It also suggested that DNA contents may changes during the seed deterioration process. All these factors significantly contributed towards the loss of seed vigor and viability in stored pea seeds.

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