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PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR MODIFICATIONS DURING THE PROCESS OF ULTRADESSICATION IN SUNFLOWER SEED

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ABSTRACT

The present study showed that germinability of sunflower seeds can be maintained for a prolonged duration if the seed internal moisture content is maintained at lower level of 3% and 4.5%. Analysis of data further revealed that seed internal moisture content has a more pronounced effect on seed germinability as compared to temperature. At low mc of 3% and mc 4.5% the viability decreased by only 4% and 21% respectfully. Whereas at high mc 7.5% and 10% mc the viability was reduced to 0 at the end of 14 years at ambient temperature. Deteriorative reactions frequently proceed in the seed more readily if the moisture content is higher, and consequently the moisture condition would constitute a threat to longevity of seed survival. The study suggested that ultra-dessication can be used as a method for safe conservation of sunflower seeds both by the scientists as well as by the farmers. At ambient storage, most species showed maximum germination when seeds were stored at moistures less than 4% with the corresponding moisture level dependent on the crop species. This value (at which germination is maximum) is within the range of the FAO/IPGRI recommendation of 5–3% moisture. Based on this data; we conclude that drying below the currently recommended seed moisture level prolongs the life spans of seeds in sunflower crops, at least under ambient storage.

Keywords: *Biodiversity, Ultradessication, Germination*

Plant biodiversity is key to the fulfilment of basic human needs but today plant biodiversity around the world is facing a great loss and one way to avoid this is to conserve the seeds in gene banks. The internationally recommended standards are that seeds be dried to 5±2% moisture content prior to sub-zero storage. Thus the moisture content and storage temperature are the two key factors affecting the longevity of seeds (1).

However, in developing countries where the costs of cold storage are prohibitive (2) low moisture content conservation (it also called ultra-dry seed storage) of seed would be a better option possible for a significant proportion of higher plants. Experiments using a number of different species have shown that longevity may be improved if seeds are dried to less than the recommended value (3, 4, 5). A lot of studies have been confirmed that ultra-dry seed storage not only can be used to maintain the quality of seeds but also improve the storability of seeds (6). Positive results of ultra-dry storage to improve storability have been reported (7, 8, 9).

The idea of ultra-drying seeds (i.e., drying to moisture contents less than 5%) was introduced as a means to reduce or eliminate the need for refrigeration. However, before committing valuable germplasm to storage at such low seed moisture, the potential benefit and risk of ultra-drying to seed longevity must be further evaluated. In the present investigations, the effect of ultra-drying of seeds to various low moisture levels on longevity was studied.

Where feasible, long-term seed storage serves as a safe and relatively inexpensive method of plant genetic resources conservation (10). Ultra-dry seed storage is a technique for decreasing the seed moisture content to less than 5% and stored at ambient temperatures, it can reduce the cost for constructing and maintaining the gene bank and has brought worldwide attention because of its potential economic effect and promising application in germplasm conservation.

This research aimed to determine whether ultra-dry storage improves the longevity of sunflower seeds. Currently seed germplasm is dried to 3–7% moisture content (mc) before storage at subzero temperatures. In the present study, seeds were dried to different low moisture contents and stored under various conditions, to identify any increase in seed longevity. Seeds were dried to 3% in sunflower (*Helianthus annus* L.). Seed conditioned to various mc's were sealed thermetically and stored at temperatures of -20°C, 10°C and ambient for 14 years. Seed germination and vigour was assessed at yearly intervals. However, reports on the protection germplasm resource and biochemical basis of seed storage in sunflower are few. Hence, our aim was to investigate seed germination ability and viability in these seeds after ultra-drying and to explore the physiology mechanism of ultra-dry storage (11).

After ultra drying some physiological indices were tested. The results indicated that Dehydrogenase, lipid peroxidation , electrical conductivity higher than those of the control seeds,. The results indicate that moisture content of seed was a key index for storage at ambient temperature (25°C) and 3.81% seem to be the best moisture content for ultra-dry seeds in our research. RAPD markers were also used to evaluate the genetic

fidelity of seeds, all RAPD profiles from ultra-dry seeds were monomorphic and similar to non-ultra-dry seeds, we conclude that variation is almost absent in ultra-dry storage. From these results, we suggest that seed moisture content less than 5% enhances longevity and ultra-dry could be an economical way for conservation of the plant genetic resource.

MATERIALS AND METHODS

Experimental Material : Freshly harvested seeds were adjusted to different moisture levels by drying over regularly regenerated silica gel in a desiccator or humidifying at 25°C for varying periods of time. Different moisture contents were achieved by removing seeds from the dessicator after intervals varying from 7–40 d, and then sealing them in laminated aluminium foil packets. Moisture contents were determined by the high low constant temperature oven methods prescribed by the International (12) and are expressed on a dry weight basis. The hermetically sealed packets were kept at -20°C, 10°C, and ambient conditions of our laboratory in New Delhi (25–45° C). To determine the effect of moisture content and temperature on seed longevity, percentage germination was evaluated at yearly intervals seeds were humidified slowly in a germinator for two days.

Seed ultra-drying treatment and pre-humidification : Seeds were packed in plastic net bags, the ratio of the seeds to silica gel was 1:5 (w/w). Seed bags were buried into silica gel in a desiccator at normal atmospheric temperature (25°C) for 15 d to reduce the moisture content of seeds to 10%, 7.5%, 4.5% and 3%. The ultra dried seeds were kept in sealed aluminium foil packages for experiment. The rapid uptake of water by dry seeds can result in imbibition injury (13).

Seeds are more likely to be damaged the lower their initial moisture content at which they imbibe water. Imbibition injury can be avoided by conditioning (humidifying) the seeds in a moist atmosphere (close to100% RH) in order to raise seed moisture contents before the seeds are set to germinate in contact with liquid water. (14).

Seed Germination Test : The primary objective of the germination test is to determine the potential of a seed to produce normal, healthy seedlings. The seeds are to be placed on suitable namely substrata, germination paper or sand germination. Seed germinability is used as an indicator of viability and various parameters are measured to assess seed vigour.

Electrical Conductivity of leachates : The electrical conductivity was measured by weighing two replicates of 10 seeds each and soaking them in 10 ml of deionised water at 25^0C for 18hours and conductivity was measured of resultant leachate water every two hours using (Control Dynamics, India) conductivity meter identifying period for achieving optimum level of conductivity, which then can be uniformly used for all conductivity experiments in sunflower seeds. The conductivity of the soaking water was measured by conductivity meter (model DDS SJ-308A, Leakage rate was expressed in l/is.cm).

Lipid peroxidation : Lipid peroxidation was measured using TBA- TCA reagent (0.5% Thiobarbituric acid in 20% trichloroacetic acid) following the method of Heath and Parker (1968). A sample of 0.25 gm of seed material was homogenized in 2 ml of distilled water and 4 ml of TBA-TCA reagent. These samples were incubated at 95^0C for 30 min. in capped reaction tubes. After incubation the samples were cooled in ice bath and centrifuged at 10,000 g for 10 min. Supernatants were collected and the (OD) was measured at 535 and 600 nm. After subtracting the absorbance of nonspecific (600 nm) from specific (535 nm) the net absorbance was expressed in terms of absorbance at 535 n mol /mg d.wt, which indicated the level of malondialdehyde (MDA) produced as a result of lipid peroxidation.

Seed protein analysis

Protein extraction : Sunflower seeds (15 seeds) were crushed with mortar and pestle to fine powder. This powdered material was transferred to test tubes to which 7 ml of defatting solvent mixture was added, mixed well and covered with aluminium foil. The composition of the defatting solution was Chloroform: Methanol: Acetone in the ratio of 2:1:1 respectively.

After 4 hrs the solvent mixture was decanted and again added 7 ml of defatting solvent mixture. This process was repeated several times until the oil was removed completely. The defatted seed powder was air-dried. The fine powdered pellet of the seed material was transferred to the eppendorf tubes for protein extraction. To 0.03 g of air-dried seed powder 150 µl of Tris: glycine buffer (pH 8.3) was added and was left overnight in the refrigerator. The sample was then centrifuged at 10,000 rpm for 15 minutes and supernatant was collected in separate eppendorf tubes.

Staining procedure : Firstly gel was fixed with fixing solution for about 30-45 min. Then the gel was stained with staining solution for about 10-12 hrs, washed and destained with distilled water several times until the band becomes visible and background became clear.

Gel electrophoresis : 0.8% agarose gel was prepared in 1 X TAE buffer. The gel was poured into the gel tray with comb placed and let it to polymerize for at least 30 min. The comb was removed and the gel was placed into the electrophoresis tank filled with 1 X TAE buffer. 25 µl of the amplified DNA was loaded into the wells. The gel was allowed to run at 90 V until the dye runs 5-6 cm from the

wells. The gel was placed in staining tray and allowed to stain with ethidium bromide stain. The gel was illuminated with UV light and photographed under trans-illuminator.

RAPD marker : DNA of seeds derived from radical and the method described by Hanania et al. (2004).For PCR amplification, eight arbitrary 10-base primers were selected for PCR amplification. Amplification reactions were performed with 25 dm^3 of 10×assay buffer, 2.0 of 1.25 mM each of dNTP's, 15 ng of the primer, 1× Taq polymerase buffer, 0.5 units of Taq DNA polymerase (TaKaRa), 2.5 mM MgCl2, and 30 ng of genomic DNA. DNA amplification was performed in a Perkin Elmer Cetus 480 DNA Thermal Cycler programmed for 45 cycles as follows: 1st cycle of 3.5 min at 92°C, 1 min at 35°C, 2 min at 72°C; followed by 44 cycles each of 1 min at 92°C, 1 min at 35°C, 2 min at 72°C followed by one final extension cycle of 7 min at 72°C. The amplification products were separated by electrophoresis in 1.2% (w/v) agarose gels with 0.5×TBE buffer, stained with 0.2 mg dm-3 ethidium bromide. A 1 kb DNA ladder was used as molecular standards and the bands were visualized and analyzed by JD-801 Gel Electrophoresis Image analytic system (Jiangsu, China). All the reactions were repeated at least twice.

Data analysis : Each band was treated as one of the marker. Scoring of bands was done from photographs. Homology of bands was based on distance of migration in the gel. Presence of band was scored as "1", absence of a band as "0" and missing data was denoted by "9".

RESULTS AND DISCUSSION

Seed Germination : The initial seed germination of fresh seeds when the experiment was 96%. After 14 years of storage at different temperature and moisture conditions the seed viability declined. Table 1.0 depicts the effect of different moisture levels at different temperatures on the seed viability. The seeds which were equilibrated to low level of moisture 3% and 4.5 % showed higher percentages of survival at all the temperatures including ambient temperature. At high moisture 7.5% and 10% and ambient temperature the seeds deteriorated with storage time. Although deterioration occurred in all the treatments at all the temperatures but the extent of deterioration was faster and maximum at ambient temperature where the seeds lost viability significantly after 14 years of storage. Seeds stored at 7.5% at ambient temperature and with 10% at all the temperatures completely lost their viability as all the seeds were dead after 14 years. The seed viability declined with storage time which was highly significant in the seeds maintained at ambient temperature compared to other temperatures of 10 C and 20 C. In case of ultra dry seeds the reduction in viability at all the temperatures was insignificant compared to the control. This clearly shows that ultra dry condition of seeds at all the temperatures performed well and viability was very close to the control (14).

Seedling length and vigour index : The initial value of shoot length was 36.75 and root length was 23.8 cm in fresh control seedlings. The root and shoot length used as a measure of vigour showed a steady decline. Both shoot and root length decreased significantly when seeds were stored at higher mc. The trends in changes of root and shoot length were similar to those for germination. Changes in shoot length were more pronounced under high mc at all the three temperatures. Amongst the treatments 3%, 4.5% mc at all the three temperature were found to be the best with a maximum root length of 12.1cm and 4.9cm respectively. A significant reduction in the vigour index was observed with storage period in all the conditions but the extent was high at ambient temperature where the reduction was highly significant. The mean vigour index of 1228 gradually decreased with increase in storage period in all the treatments. The change in vigour index is closely followed by changes in viability. Storage under high mc (7.5%) and ambient temperature resulted in a significant fall in the vigour index (643) after 14 years of storage.

Dehydrogenase activity : Activity of enzyme dehydrogenase declined with seed deterioration .In fresh seeds the activity of enzyme dehydrogenase (OD/g fresh wt) was found to be 0.8549. Similar activity has been observed in the seeds maintained at 3%, 4.5% mc at all the three temperatures after 14 years of storage. However, at ambient temperature the activity was significantly lowered when seeds were stored with 7.5% and 10% of mc. However the decline in activity was less pronounced at 4.5% mc at all the temperatures. Moisture content seems to have more deleterious effect on dehydrogenase activity compared to temperature .Further appraisal revealed that in spite of having same germination percentage of various treatments there was variation in their dehydrogenase enzyme activity. This could probably because of the internal state or the vigour status of the seed which has not been reflected by the germination percentage of the seeds.

Lipid peroxidation : Sunflower being an oil rich seed the extent of lipid peroxidation is of greater importance in the context of its storability pattern. Result of the data on lipid peroxidation showed that the values were least in healthy and 97 % viable seeds and showed and increased trend with seed deterioration. The values of lipid peroxidation were found to be significantly different under different treatments which increased with seed deterioration. The low value of 0.0954 malionaldehyde content (n mol/mg dwt.) in fresh control increased to 15-18 times.

The SDS –PAGE protein profile : The SDS –PAGE protein profile did not show significant changes except that

some of the high mobility low molecular weight bands disappeared during deterioration. The sharp bands disappeared or their intensity decreased as the viability decreased. The alteration in the banding pattern might be due to the loss of low molecular weight proteins under deterioration at ambient temperature and high moisture. In ultra dry seeds there were only minor changes of one or two bands in the protein banding pattern.

Monitoring of genetic fidelity by RAPD : In order to confirm genetic fidelity (at molecular level) of seeds after ultra-dry treatment, the seeds were screened with the 8 random RAPD primers, one primer that produced distinct amplification profiles. The representative profile of the ultra-dry seeds and the control (non-ultra dry) with primer is shown in Figure. It was obvious that the ultra-dry seeds showed identical RAPD profiles (i.e. nopolymorphism was observed). These results confirmed the genetic fidelity of the ultra-dry seeds.

Several studies on the effect of seed deterioration at DNA level pointed out that chromosomal aberrations, point mutations and decrease in the activity of DNA repair enzymes are some of the major events , occurring during

Table-1 : Effect of Moisture and Temperature on Germination of Sunflower Seeds.

Moisture in %		No. of seeds germinates out of 25.		
		LTS	MTS	Ambient
3	R1	22	21	22
	R2	23	22	21
	R3	23	23	23
	R4	23	22	21
	%	91	88	87
4.5	R1	22	22	18
	R2	23	23	23
	R3	23	22	15
	R4	22	20	13
	%	90	87	69
7.5	R1	22	19	—
	R2	21	21	—
	R3	20	21	—
	R4	20	20	—
	%	83	83	

Table-2 : Effect of Moisture and Temperature on seedling length.

Moisture in %		Seedling length in cm.		
		LTS	MTS	Ambient
3	R1	13.7	21	22
	R2	13.3	22	21
	Mean	13.5	23	23
4.5	R1	10.1	10.3	9.0
	R2	11.0	10.5	7.9
	Mean	10.55	10.5	8.45
7.5	R1	8.5	8.3	—
	R2	7.0	7.2	—
	R3	7.75	7.75	—
10	R1	—	—	—
	R2	—	—	—
	Mean	—	—	—

Table-3 : Effect of Moisture and Temperature on Vigour Index.

Moisture in %	Vigour Index.		
	LTS	MTS	Ambient
3	1228	1161	1174
4.5	949	904	583
7.5	643	643	0
10	0	0	0

Table-4 : Effect of Moisture and Temperature on Electric Conductivity test.

Moisture in %		Seedling length in cm.		
		LTS	**MTS**	**Ambient**
3	R1	1.44	1.21	1.90
	R2	1.21	1.37	1.58
	Mean	1.325	1.29	1.74
4.5	R1	1.33	1.39	2.25
	R2	1.88	1.04	1.65
	Mean	1.605	1.215	1.95
7.5	R1	1.32	1.50	2.38
	R2	1.31	1.29	2.13
	R3	1.315	1.395	2.255
10	R1	1.24	1.85	2.39
	R2	1.17	1.79	1.98
	Mean	1.205	1.82	2.185

Table-5 : Effect of Moisture and Temperature on Enzyme Dehydrogenase.

Moisture in %		Seedling length in cm.		
		LTS	**MTS**	**Ambient**
3	R1	0.9790	0.6510	0.2934
	R2	0.7308	0.5403	0.2048
	Mean	0.8549	0.5956	0.2491
4.5	R1	0.2573	0.4528	0.1081
	R2	0.6845	0.3646	0.1326
	Mean	0.4709	0.4087	0.1203
7.5	R1	0.6207	0.3057	0.2542
	R2	0.7768	0.3027	0.2988
	R3	0.6987	0.3042	0.2765
10	R1	0.3759	0.2588	0.1378
	R2	0.2846	0.1310	0.1648
	Mean	0.3302	0.1949	0.1513

Table-6 : Effect of Moisture and Temperature on the Lipid Peroxidation.

Moisture in %		Difference of 600-532 nm wave length		
		LTS	**MTS**	**Ambient**
3	R1	0.1008	0.1145	0.10865
	R2	0.0900	0.0906	0.0950
	Mean	0.0954	0.1025	0.1018
4.5	R1	0.0949	0.1016	0.1078
	R2	0.1078	0.1089	0.1095
	Mean	0.1013	0.1052	0.1086
7.5	R1	0.0481	0.0950	0.1013
	R2	0.0977	0.1191	0.1016
	R3	0.0729	0.1070	0.1014
10	R1	0.0976	0	0
	R2	0.0854	0	0
	Mean	0.0915	0	0

the process of ageing in seeds(McDonald, 1999). While RAPD markers would scan the entire genome at random irrespective of being expressed or non–expressed, it is possible that most of the polymorphism observed in the amplified fragments are interspersed in the regions which are not highly conserved. Hence these sectors are likely to be fragmented or altered more at random than the regions which are highly conserved. In the present study

Table-7 : Effect of Moisture and Temperature on SDS-PAGE.

Band no.	M.Wt (Kda)	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	250	+	-	-	-	-	-	-	-	-	-	-	-	-	-
2	130	+	-	-	-	-	-	-	-	-	-	-	-	+	-
3	95	+	-	-	-	-	-	-	-	-	-	-	-	+	-
4	72	+	-	-	-	-	-	-	-	-	-	-	-	+	-
5		-	+	+	+	-	+	+	+	+	+	+	+	+	+
6		-	+	+	+	+	+	+	+	+	+	+	+	+	+
7	55	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8		-	+	+	+	+	+	+	+	+	+	+	+	+	+
9		-	+	+	+	+	+	+	+	+	+	+	+	+	+
10	36	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11		-	+	+	+	+	+	+	+	+	+	+	+	+	+
12	28	+	+	+	+	+	+	-	-	-	-	-	+	-	-
13		-	+	+	+	+	+	+	+	+	+	-	+	+	+
14	17	+	-	-	-	-	-	-	-	-	-	-	-	-	-
15		-	+	+	+	+	+	+	+	+	+	+	+	+	+
16	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17		-	+	+	+	+	-	-	-	+	+	+	-	+	-

Score of protein bands present in the different treatments at different temperature.
Here,
Band present = + (positive sign)
Band absent = - (negative sign)
Band no.=1 to 17.
Treatment=1 to 14.Treatment no. 1=Ladder.

1 2 3 4 5 6 7 89 10 11 12 13 14

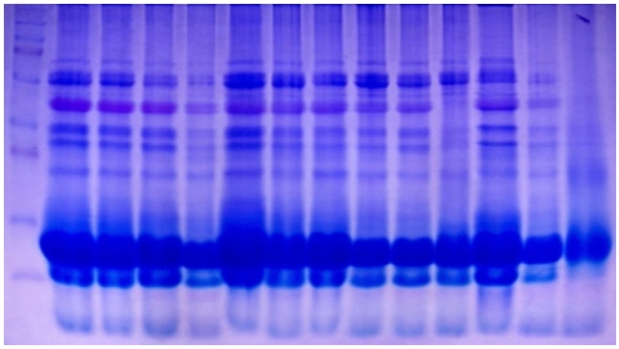

Fig.-1 : Photograph of SDS-PAGE gel.

Lane 1-14:-1-ladder, 2-control, 3-3%LTS, 4-3%MTS, 5-3%Ambient, 6- 4.5%LTS, 7- 4.5% MTS, 8- 4.5%Ambient, 9-7.5%LTS, 10- 7.5%MTS, 11-7.5%Ambient, 12-10%LTS, 13 10%MTS,

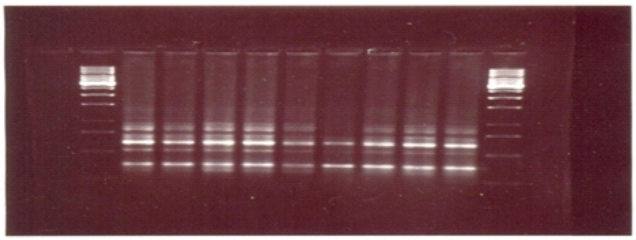

Fig.-2 : Photograph of RAPD Gel.

there was not much difference in the RAPD profile of the aged and control seeds .The RAPD clustering showed the possible accumulation of aberrations in non-coding part, the junk DNA.

Temperature and moisture content has been indicated to be important factors, which influences the seed longevity during processing and storage of seeds. Therefore, the present investigations on sunflower has been undertaken to understand the effect of different temperatures and mcs on the seed storability. The present study showed that germinability of sunflower seeds can be maintained for a prolonged duration if the seed internal moisture content is maintained at lower level of 3% and 4.5%. Analysis of data further revealed that seed internal moisture content has a more pronounced effect on seed germinability as compared to temperature. At low mc of 3% and mc 4.5% the viability decreased by only 4% and 21% respectfully. Whereas at high mc 7.5% and 10% mc the viability was reduced to 0 at the end of 14 years at ambient temperature. Deteriorative reactions frequently proceed in the seed more readily if the moisture content is higher, and consequently the moisture condition would constitute a threat to longevity of seed survival.

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