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RESEARCH ARTICLE

STUDIES ON MEMBRANE PERMEABILITY, LIPID PEROXIDATION ACTIVITY AND SDS-PAGE FOR IMPROVED GERMINABILITY AND FIELD PERFORMANCE OF INVIGOURATED HIGH MEDIUM VIGOUR SOYBEAN SEED

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ABSTRACT

Five-month old stored soybean seeds (*Glycine max* [L.]Merr., cv. Soyamax) were subjected to dry and wet treatments. Dry treatments were done with powdered crude plant materials viz. red chilli powder @1g/kg of seed, neem leaf powder @2g/kg of seed, chemicals viz. iodinated calcium carbonate @ 2g/kg of seed (30mg iodine impregnated to 2g calcium carbonate), *para*-amino-benzoic acid @500mg/kg of seed, ferulic acid @500mg/kg and pharmaceuticals viz. aspirin @50mg/kg of seed. In case of wet treatments, Soaking-Drying (S-D), Moist-Sand-Conditioning-Drying (MSC-D), Moist-Sand-Conditioning-Soaking-Drying (MSC-SD) were employed with dry treatments. The results revealed that, wet treatment, MSC-SD followed by dry treatments with aspirin and neem leaf powder significantly improved the germination percentage, total seedling length, vigour index, field performance and productivity. Soaking-drying showed adverse effect on germinability probably due to soaking injury. Physiological and Biochemical studies indicate that MSC-SD followed by aspirin, neem leaf powder and iodinated calcium carbonate showed reduced leakage of electrolytes, sugars, amino acid with lower lipid peroxide formation than the control. It has also been noted in SDS-PAGE analysis that the invigorated seeds, especially MSC-SD showed higher number of polypeptide bands with high banding intensity than the control. On the basis of the observation, MSC-SD may be recommended for the improvement of storability as well as field performance and productivity of high medium vigour soybean seeds.

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INTRODUCTION

Soybean (*Glycine max* [L.] Merr.) is the world's most important seed legume, which contributes to 25% of the global edible oil, about two-thirds of the world's protein concentrate for livestock feeding. Soy belongs to the *fabaceae* or *leguminosae* (legume) family which is the third largest family of flowering plants, with well over 19,000 distinct species. It is one of the few plants that provide a complete protein, and is therefore often used as a substitute for meat and dairy products. Orthodox seeds are characterized by their ability to tolerate desiccation and to retain their viability for a long time in the dry state. The rate at which seeds lose vigour during storage is affected by environmental factors such as temperature, moisture, and O_2/CO_2 concentrations and their viability may go down to below 50% in the next sowing season.

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Several comprehensive reviews have identified free radical-mediated lipid peroxidation, enzyme inactivation or protein degradation, disruption of cellular membranes, and damage to genetic (nucleic acids) integrity as major causes of seed ageing. Biochemical deterioration during seed ageing has been studied mostly under accelerated ageing conditions using high temperature and high seed water content. Under such storage conditions, seeds typically lose their viability within a few days or weeks. Hydration-dehydration treatments are effective only when given to medium vigour seeds and are not suitable for high-vigour seeds. But the soaking drying treatment in leguminous seeds would adversely affect germinability due to soaking injury (Saha, Mandal and Basu, 1990). In this situation, a number of inexpensive non-toxic dry physiological seed treatments have been developed in the present laboratory by Mandal and co-workers (Mandal *et al.* 2000; De *et al.,* 2003 and Guha *et al.,* 2012). The present study has been taken up to evaluate the efficacy of dry and wet seed treatments for the maintenance of germinability and improved productivity of medium vigour soybean seeds.

MATERIALS AND METHODS

Treatments were given to 5-month-old high medium vigour soybean seeds which were stored in the rubber stoppered glass bottles under ambient conditions. Seeds were subjected to dry treatments viz., aspirin (active ingredient *ortho*-acetyl salicylic acid, @50mg/kg of seed), iodinated calcium carbonate (30mg iodine impregnated to 2g calcium carbonate @ 2g/kg of seed), red chilli powder (active ingredient, capsaicin @1g/kg of seed), neem leaf powder (active ingredient, azadirachtin @2g/kg of seed), *para*-amino-benzoic acid (500mg/kg of seed), ferulic acid (500mg/kg of seed) under ambient condition (temperature 28°C). After dry dressing, bottles were thoroughly shaken for the mixing of chemicals and crude plant powders with the seed and this process was continued for at least another 7 days. For wet treatment, viz., Soaking-Drying seeds were soaked in double volume of water for 2 hour followed by drying to its original moisture content under the sun or artificial dehumidified drying cabinet for 2-3 days. Moist sand conditioning of seeds were done firstly by pre-moistening the air dry sand by 5% moisture (seed: sand:: 1:3) in the container and then seeds were thoroughly mixed with the moist sand and then kept covered for 24 hour. After the stipulated period, seeds were sieved to let the sand pass and then dried to its original moisture content. In case of Moist Sand Conditioning Soaking Drying, after Moist Sand Conditioning for 24hour followed by soaking in water for 2 hour and then drying back to its original moisture content. All the treated seeds along with control were then stored in the desiccators containing fused calcium chloride for 7 days for moisture stabilization to a uniform level. After 30 days, treated and untreated seeds were subjected to accelerated ageing at 93% relative humidity (RH) and 40°C temperature for 18 days. Germination tests of treated and untreated seeds were conducted immediately after treatment and after ageing following the inclined glass plate blotter method of Punjabi and Basu (1982). Before germination, seeds were slurry dressed with fungicide to control fungal growth during germination.

To study the membrane permeability as evidenced by electrical conductance, 10 seeds of each treatment were soaked in 30 ml of distilled water for 30 minutes at room temperature (28±1°C) and then Electrical Conductance (E.C.) of seed leachate was recorded in a systronic conductivity bridge, following the method of Anderson *et al*. (1964). The amount of leaching of sugar leached out from the seed was determined following the method of McCready *et al*. (1950) with minor modification. Pre-cooled 2 ml leachate of soybean seed was taken in a hard glass test tube separately. Then 4 ml of ice-cold freshly prepared Anthrone reagent (0.2% anthrone in 95% H_2SO_4) were added and kept in cold for 30 minutes for the development of bluish green colour and the intensity of colour was then measured by systronic spectrophotometer at 580 nm. The amino acid estimation was conducted following the method of Moore *et al.* (1948), Misra *et al.* (1975) with minor modification. Ten seeds of each treatment were soaked in 30 ml of distilled water for 3 hour 30 minutes at room temperature (28±1°C) and then 0.1 ml leachate was taken in a hard glass test tube and 1 ml of 2% ninhydrin solution was mixed thoroughly. The volume was made up to 2ml with distilled water and placed in a boiling water bath for 20 minutes. 5ml of diluents (water: n-propanol 1:1) was added to it and the intensity of colour was then measured by systronic spectrophotometer at 570 nm. Lipid peroxide formation of treated and untreated seeds was estimated following the method of Bernheim *et al.* (1948) with minor modifications. Five ml of 1% TBA reagent and 2ml of 1N sulphuric acid was added to 100 mg tissue. The mixture was heated for 15 minutes in a water bath at 100°C. After cooling 5ml of 2-methoxy ethanol were added and shaken and then the mixture was centrifuged at 10,000rpm for 10 minutes and the intensity of colour was then measured by systronic spectrophotometer at 520 nm. To study the SDS-PAGE of total soluble protein was carried out by using 12% acryl amide gel according to the method prescribed by Laemmli (1970). 100 mg imbibed tissue was taken in pre-cooled mortar pestle and crushed in 2 ml of crushing buffer and centrifuged at 14000 rpm for 30 minutes.. After TCA-Acetone precipitation the supernatant was collected for protein estimation by Lowry *et al.* (1951) and 80 µg equivalent protein was used from each sample for loading on to the gel. A constant current of 25 mA was applied until the tracking dye crossed the stacking gel. Then a constant voltage of 80 volt was applied until the tracking dye reaches bottom of the resolving gel. Then the gel was stained using coomassie brilliant blue, R 250 for 6 hours and destained using a mixture of 100 ml methanol, 100 ml acetic acid and 800 ml of distilled water until the band were clearly visible. Field experiment was conducted at Calcutta University Agricultural Experimental Farm, Baruipur, South 24-paraganas during rabi season in the year 2014 and 2015 using RBD with 3 replications for each treatment measuring each plot to be $10m^2$. Fertilizer dose of $N:P_2O_5:K_2O$ was added at the ratio of 20:40:40 kg/ha. During final land preparation, the whole amount of phosphate, potassium and 50% nitrogen was added. The rest of the nitrogen was added in two split doses, one at 30 days after sowing and another at flower initiation stage. The post sowing irrigation was done on the same day. Besides, three more irrigations, weeding and pesticide application (chlorpyrifos) was done time to time throughout the cropping period. Field emergence data was taken when the plant reaches upto 25cm in height. Plant height, number of branches, number of pod per plant, pod weight, seed yield and 1000 seed weight were recorded after harvesting. The data obtained from the laboratory germination test and biochemical tests were analysed statistically to evaluate the treatment effects on vigour and viability of soybean, following the method of analysis of variance (Fisher, 1948). Germination percentage data of treated and untreated seeds were transformed to their respective arc-sin values before computation and root and shoot length data were analysed as such.

RESULTS AND DISCUSSION

Germination test conducted immediately after treatment did not show any significant difference on germinability between treated and untreated seed (Table 1). But after accelerated ageing at 93% RH and 40°C for 18 days, wet treatment such as moist-sand-conditioning-soaking-drying (MSC-SD) and moist-sand-conditioning-drying (MSC-D) showed significant improvement on germinability over untreated control. The soaking-drying treatment (SD) showed adverse effect on germinability due to soaking injury during hydration.

Table 1. Effect of seed invigoration treatments on germinability of high medium vigour soybean immediately after treatment i.e., before ageing and after accelerated ageing at 93%RH and 40°C for 18 days

Treatment	Before Ageing					After Ageing				
	Germination		Mean root length (mm)	Mean shoot length (mm)	Vigour index	Germination		Mean root length (mm)	Mean shoot length (mm)	Vigour index
	%	Arc-sin value				%	Arc-sin value			
Control	100	90	167.0	103.3	27015	46.5	42.97	92.8	66.4	7403
Aspirin	100	90	175.5	111.7	28700	65.5	54.50	131.3	89.2	14443
Iodinated calcium carbonate	100	90	167.3	109.3	27655	60.0	50.83	123.3	80.3	12216
Red chilli powder	100	90	168.4	104.9	27325	58.5	49.92	125.9	82.2	12174
Neem leaf powder	100	90	172.3	106.9	27925	60.5	51.18	127.3	80.5	12572
Para amino benzoic acid	100	90	174.6	102.7	27700	45.0	42.13	102.0	61.8	7371
Ferulic acid	100	90	175.2	110.7	28580	51.0	45.58	110.7	70.6	9246
S-D	98.5	83	167.0	98.8	26228	23.5	28.78	81.4	60.1	3325
MSC-D	100	90	169.4	101.3	27045	71.0	58.01	135.5	86.6	15769
MSC-SD	100	90	171.2	108.2	27935	72.0	58.94	139.7	88.9	16459
C.D at 0.05 P	NS	NS	NS	NS	-	19.1	-	11.7	10.4	-

Data on germination percentage and seedling length were recorded after germination for 7 days at 28 ± 1ºC temperature. Abbreviations: SD = Soaking-drying; MSC-D= Moist-sand-conditioning-drying; MSC-SD = Moist-sand-conditioning-soaking-drying; VI (Vigour Index) = Germination% X Seedling length; CD = Critical Difference; NS=Non Significant

Table 2. Efficacy of seed invigoration treatments on field performance and productivity of stored high medium vigour soybean

Treatment	Field Emergenc(%)	Plant height(cm)	No.of branches/ plant	Length pod(mm)	No.of pod/ plant	Seed yield/m^2(g)	1000 seed weight(g)
Control	62	99.7	2	17.6	19	218.4	123.9
Aspirin	65	111.1	2	18.1	35	238.1	133.7
Iodinated calcium carbonate	63	105.3	1	17.8	29	221.6	125.5
Red chilli powder	64	106.2	1	18.5	30	220.7	124.7
Neem leaf powder	62	105.9	3	17.4	28	221.1	125.2
Para amino benzoic acid	63	108.0	2	18.3	31	232.2	130.8
Ferulic acid	64	108.4	2	17.9	26	239.4	134.4
S-D	60	93.4	1	16.7	13	200.4	114.7
MSC-D	64	100.6	2	17.1	31	228.8	129.1
MSC-SD	66	112.3	2	18.8	34	247.6	138.6
C.D at 0.05 P	NS	NS	NS	NS	2.8	2.5	1.3

Data on field emergence and seed yield along with yield attributes were recorded after 30 days and 110 days respectively.

Table 3. Effect of seed invigoration treatments on membrane permeability,lipid peroxidation activity and total soluble protein of soybean immediately after treatment i.e., before ageing and after accelerated ageing at 93%RH and 40°C for 18 days

	Before Ageing					After Ageing				
	Electrical Conductance (μScm^{-1})	Leakage of sugar (µg glucose eqiv./ml)	Leakage of amino Acid (µg leucine eqiv./ml)	Lipid Peroxidation (nM/gm of fresh W.)	Total soluble protein (µg/gm of tissue)	Electrical Conductane (μScm^{-1})	Leakage of sugar (µg glucose eqiv/ml)	Leakage of amino acid (µg leucine eqiv/ml)	Lipid peroxidation (nM/gm of fresh W.)	Total soluble protein (µg/gm of tissue)
Control	26.1	31.3	28.8	78.9	158.5	50.6	80.5	53.5	110.7	271.7
Aspirin	26.2	31.3	28.7	78.5	158.7	35.1	50.7	57.1	102.1	266.7
Iodinated calcium carbonate	26.1	31.4	28.6	78.1	158.3	36.4	64.1	64.3	100.6	243.3
Red chilli powder	26.2	32.1	28.6	78.6	158.6	55.7	73.1	57.2	126.2	258.3
Neem leaf powder	26.1	32.2	28.7	78.7	158.3	36.9	61.1	64.2	103.7	218.3
Para amino benzoic acid	26.2	33.1	28.7	78.9	158.6	40.9	61.1	64.3	114.5	308.3
Ferulic acid	26.1	33.2	28.5	78.9	158.3	40.6	65.6	50.1	112.2	213.3
S-D	26.2	33.2	28.9	79.7	158.3	55.1	119.4	71.4	147.1	316.7
MSC-D	26.1	32.7	28.4	78.1	158.3	33.9	49.2	53.6	98.3	200.3
MSC-SD	26.1	31.2	28.1	77.7	158.1	31.2	38.8	41.4	97.5	190.1
C.D at 0.05 P	NS	NS	NS	NS	NS	1.04	2.5	17.8	3.9	10.2

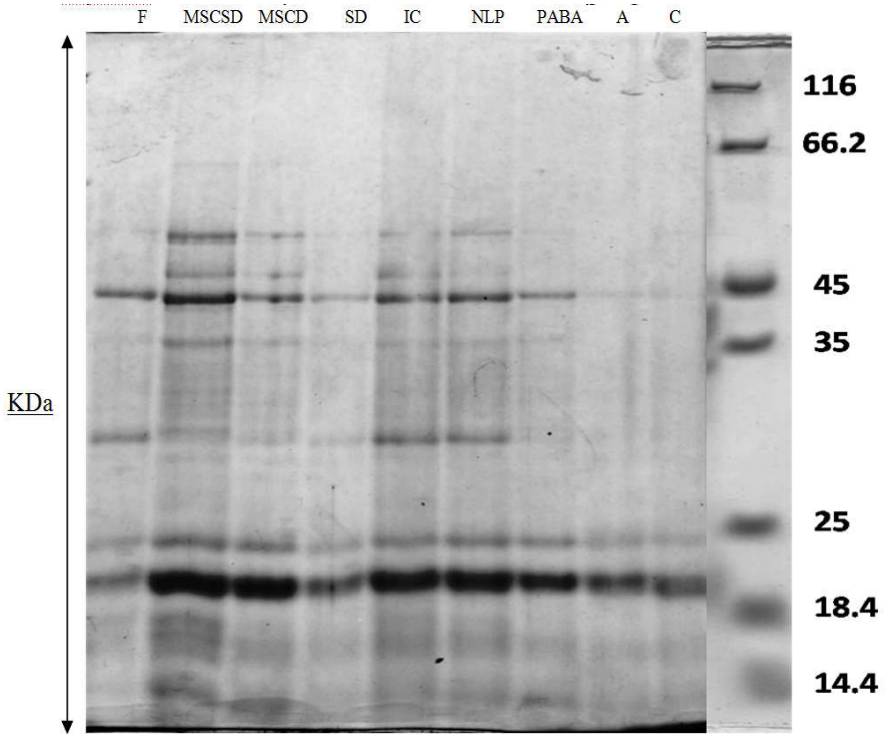

Fig. 1.Peptide profile of treated and untreated seeds of high medium vigour soybean (cv. Soyamax) immediately after treatment i.e., before ageing

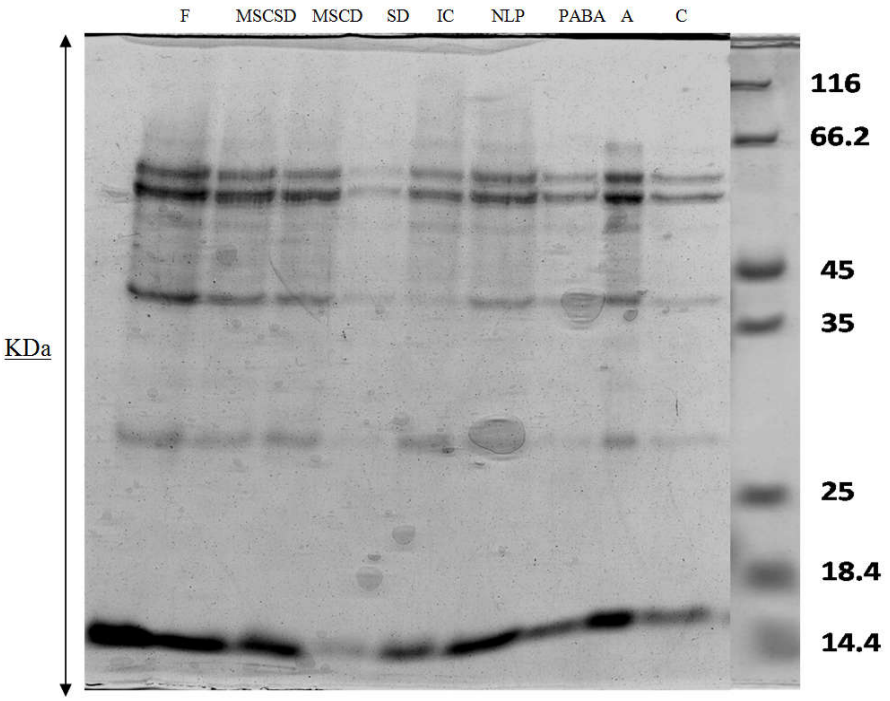

Fig. 2. Peptide profile of treated and untreated seeds of soybean (cv. Soyamax) after accelerated ageing at 93% RH and 40°C temperature for 18 days

Among the dry treatments, aspirin followed by neem leaf powder and red chilli powder also showed significant improvement on vigour than the control. The crop raised from the mid-storage treated and untreated seeds revealed that there was a marginal difference between treated and untreated seeds on field emergence, plant height, number of branches per plant and length of pod (Table 2).

But the wet treatment MSC-SD showed significant increase in yield per unit area and 1000 seed weight over untreated control and SD showed adverse effect due to soaking injury (Table 2). Among the dry treatment, aspirin ferulic acid and *para*-amino benzoic acid has also shown better results in improving yield and other yield attributes. The membrane integrity was estimated by electrical conductance, leaching of sugar and amino acid as well as production of lipid peroxide formation

did not show any significant diiferences between treated and untreated seeds when tested immediately after treatment (Table 3). But after accelerated ageing at 93% RH (relative humidity) and 40°C temperature for 18 days, leaching of electrolytes, sugars, amino acid and lipid peroxidation were significantly lowered in MSC-SD and MSC-D along with few dry treated seeds than the untreated control. Among the dry treatments, aspirin, iodinated $CaCO_3$ and neem leaf powder has shown greater membrane integrity than the control (Table 3). The wet treatment, soaking-drying showed reduced leakage of electrolytes, sugars and amino acid than the control inspite of lower germination, probably due to initial leakage at the time of soaking during treatment. Soluble protein content and peptide profile of 5-month old high vigour soybean seeds after subjecting to accelerated ageing at 93% RH and 40°C for 18 days showed that band intensity, band number or disappearance of some bands with the advancement of ageing. Polypeptide bands of diverse molecular weight ranging from 14.09 KDa to 79.39 KDa was observed in case of 5 month old treated and untreated seeds under before ageing conditions (immediately after treatment) and polypeptide bands of diverse molecular weight ranging from 13.57 KDa to 68.48 KDa was observed in case of accelerated ageing. Moist-sand-conditioning-soaking-drying treatment showed maximum resolved peptide bands i.e. 19 with 32.07 KDa band intensity, while seed treated with soaking drying had 6 peptide bands and the untreated control had 7 peptide bands. The mode of action of hydration-dehydration treatments have been discussed by several workers (Basu, 1994; Mandal *et al.* 2000; Saha *et al.* 1990). Two possibilities, viz.,

- Involvement of cellular repair system in correcting age-induced biochemical lesions during seed hydration (Villiers and Edgumbe,1975; Burgass and Powell,1984)
- Counteraction of free radical and lipid peroxidation reactions by hydration-dehydration treatments (Berjak1978; Mandal *et al.* 2000; Saha *et al.* 1990).

High lipid peroxidation and oxidative stress have been observed during storage of various seeds and have been widely proposed as the major cause of deterioration during seed aging (Wilson and McDonald, 1986b; McDonald, 1999; Pukacka and Ratajczak, 2005) Accelerated seed ageing also resulted in increased lipid peroxidation (Kumar and Knowles, 1996; Chiu *et al.*, 1995; Hsu *et al.*, 2003). It has been known that the reactive oxygen species (ROS), major cause of lipid peroxidation in cell membranes, can be generated not only in metabolism during stress and ageing, but also in metabolism of a plant under normal conditions (Kumar and Knowles 1996). In conformity our results also showed that lipid peroxidation may be cause of seed deterioration in soybean. Regarding the mode of action of the dry seed invigoration treatments, the role of iodine in the stabilization of double bonds of unsaturated fatty acids moieties of lipoprotein biomembranes as a possible reason for viability extension was suggested by Basu and Rudrapal (1980). Aspirin is an anti-inflammatory drug and chemically, it was a weak acid. It may decrease the production of free radicals and superoxide and may interact with adenyl cyclase to alter the cellular concentration of cAMP (Bertrum, 1998). To maintain vigour and viability by dry treatment, Umarani *et al*. (1997) showed the efficacy of leaf powder (*Albizzia amara*, *Vitex negundo* and *Azadirachta indica*) treatment on *Casuarina equisetifolia* seeds. Electrophoresis of proteins is a powerful tool for identification of genetic diversity and the SDS-PAGE is particularly considered as a reliable technology because seed storage proteins are highly independent of environmental fluctuations. The alteration in banding pattern of protein profile of aged seeds compare to that of fresh seed was established through disintegrating higher molecular weight peptide bands into lower sub units. Machado *et al.* (2001) reported that when seeds were placed at high relative humidity and temperature, there was a change in protein electrophoretic pattern as increased moisture is enough to damage the seed on its structure. Vishwanath *et al.* (2007) revealed that due to accelerated ageing there has been a decline in soluble protein banding pattern in terms of band intensity, band numbers or even total loss of some bands. Kehinde *et al.* (2013) in amaranthus, Vasudevan *et al.* (2012) in pea nut and Das *et al.* (2010) in rice reported similer changes in number and intensity in protein banding of low vigour seeds. On the basis of the present results, wet treatments with moist-sand-conditioning-soaking-drying (MSC-SD) and moist-sand-conditioning-drying (MSC-D) may be recommended for improved germinability and field performance of high medium vigour soybean seeds.

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