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Limitation of Improvement in Germination by Osmopriming of Differentially Aged Non-Orthodox Neem (*Azadirachta indica*) Seeds

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Abstract

This work involves physiological and biochemical features of seed ageing gauged from seed viability and vigour over the period of storage. Both conventional storage (natural ageing) and controlled deterioration (accelerated ageing) are resulted in loss of germination capacity and vigour as well as poor seedling establishment. Present findings indicate that both natural and accelerated ageing sustain similar pattern, except their mortality curve. In natural ageing, prior to entering sigmoidal type decline a period of relative stability exists; whereas in accelerated aging, such relative stability is absent. It is also observed that rate- controlling process of ageing (natural ageing slow whereas accelerated ageing fast) was dependent upon moisture content and temperature. These physical factors have negative linear correlation with seed viability. Membrane integrity and lipid peroxidation are associated with seed ageing, however peroxidation does not hold exact with accelerated ageing. Additionally, these aged seeds were exposed to osmopriming (controlled hydration) resulted in improved germination characterized by faster and uniform germination. For the first time, it was strongly established that osmopriming significantly improves the seed germination (about 12-17%) until a critical level (up to 50% germination) during the ageing and thereafter priming does not support the process of improvement of germination. Such improvement prediction is important for physiologists and seed technologists to recruitment the degree of priming.

Keywords: Natural ageing; Accelerated ageing; Osmopriming; Germination improvement; *Azadirachta indica*; Membrane integrity; Lipid peroxidation

Abbreviations: PEG: Polyethylene Glycol; MC: Moisture Content; TTC: Tri-phenyl Tetrazolium Chloride; LSMC: Lowest Safe Moisture Contents; MDA: Malondialdehyde; TBARS: Thiobarbituric Acid-Reactive Substances; BHT, Butylated Hydroxy Toluene; DM: Dry Mass; FW: Fresh Weight

Introduction

The importance of tropical tree species is wildly recognized. Many tropical trees propagate through seeds. It is a matter of concern, when the seeds of some of the tropical trees have low to very low storage longevity. These seeds generally display intermediate or recalcitrant storage behavior. Unlike orthodox, seeds [intermediate or recalcitrant] shed at very high moisture contents do not survive below critical moisture content (depend upon drying rate and condition). Intermediate seeds survive drying and/or moderate low moisture content, but are often injured by low temperature. This is attributed to their sensitivity to desiccation and/or low temperatures. Storage of such seeds results in loss of seed viability under natural condition by way of decline in the moisture content. These seeds are to be conserved (stored) for sake of reforestation as well as *ex situ* conservation as forest genetic resources. Neem (*Azadirachta indica),* is a valuable and economically important tropical tree species. The seeds of *Azadirachta indica* have been characterized as having intermediate storage longevity. They lose viability within 3 months after harvesting [1,2]. Loss of germinability occurs during dry storage over the time. According to Heydecker et al. [3] seed ageing exhibits deteriorative changes that lead to decreased viability, poor germinability and weak seedling establishment. Besides natural ageing (NA), accelerated ageing (AA) under high temperature and high humidity have a great potential for understanding the mechanism of ageing and associated deterioration processes of seed [4]. The process of deterioration under accelerated ageing conditions is considered fundamentally similar to those under normal condition but not so far verified. However, the major difference is that the rate of deterioration is much faster during accelerated ageing. A number of studies have been carried out in the past to analyze the physiological and biochemical changes associated with accelerated ageing in different seeds [5-7]. Membrane integrity is important marker to determine seed longevity. It is most probable site of biochemical and biophysical changes. Membrane chemical stability is determined by degree of peroxidation of membrane lipids leading to irreversible gel phase domains and loss of membrane function. During last few decades, several priming treatment established to improve germination time, rate, homogeneity and synchrony of aged seeds. Osmopriming is a pre-sowing treatment that exposes seeds to such osmoticum that allows partial hydration but prevent germination [8]. During priming water uptake is controlled by the lowering of the water potential of pre-treated solution with an inert osmoticum. Such primed seeds tend to have an improved seed performance indicated by better germination rate and uniformity [9]. Polyethylene glycol-6000 is often used as the osmopriming reagent [10]. If the seeds are not used immediately after treatment, then they must be dried back to lowest safe moisture contents (LSMC) at which they can be stored without deterioration. Present work depicts ageing dynamics in both natural and accelerated aged seeds. It is not known whether mechanisms of seed ageing are alike under accelerated ageing and natural ageing. Neem seeds behave as intermediate storage longevity. That gives the opportunity to execute

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natural and accelerated ageing at static point. For the first time to the best of our knowledge, this article is also an attempt to determine the limits for germination improvement of aged seed under the influence of osmopriming.

Materials and Methods

Seed collection and extraction

Matured and ripened yellow fruits of *Azadirachta indica* (Neem) were collected manually from the trees growing on the campus of Pt. Ravishankar Shukla University, Raipur, (21°14'14" N latitude and 81°38'55" E longitudes) and brought to laboratory immediately. The initial moisture content of seeds was determined using randomly chosen mechanically depulped seeds. Seeds with endocarp were extracted from the fruit tissue by soaking them in 20% H_2SO_4 (Merck, India) for 30 min following depulping by rubbing over a wire mesh and washing repeatedly under tap water. Seeds with hard endocarp were dried to their initial moisture content under shade for storage [2,11]. Seeds were harvested from hard endocarp by breaking gently to analyze physiological and biochemical variables after every 15 days after storage.

Natural and accelerated ageing treatment

Seeds were stored single layered in well-aerated basket in laboratory conditions (temperature 26°-28°C and RH 45-50%) for natural ageing process. For accelerated ageing treatment, seeds were placed in 18 × 10 × 2 cm closed plastic cabinet at 45°C with a fully saturated humid atmosphere (100% RH) as Delouche and Baskin [4] method for various time intervals 1.5 h, 3 h, 4.5 h, 6 h, 9 h, 12 h, 15 h and 24 h. The seeds were collected at different germination percentage and denoted as G100%, G90%, G70%, G50%, G30% and G0% from natural aged (NA) and accelerated aged (AA) lots. Seeds with 100% germination are referred as control.

Optimization of osmopriming treatment

Ten average weighted seeds (G100%) were placed in each 12 cm diameter petri dish with single layer of filter paper moistened with 5ml of different concentration of PEG-6000 for -0.21, -0.27, -0.58 and -0.78 MPa for 5 days to find the PEG concentration at which seed germination is delayed or prevented. Seed germination was completely excluded at -0.58 and -0.78 MPa PEG concentration (Figure 1). Additional, ten average weighted seeds (G100%) were placed with water as control and were tested for germination. A three-way factorial experimental design was used to compare priming conditions for optimization of osmopriming for germination improvement. Seeds with 60% germination were surface-sterilized and placed at 15°C and 30°C in dark for 1, 2 and 3 days at -0.58 and -0.78 MPa PEG-6000 solutions. The optimal condition was gauged from germination percentage and germination index/ germination vigour. This leads to optimal osmopriming protocol for germination [priming seeds with -0.78 MPa PEG-6000 at 30°C for one day] (Supplementary Tables 1 and 2).

The osmopriming treatments of both aged (AA and NA) seeds were carried out in 12 cm diameter petri dish with single layers of filter paper moistened with 5 ml of -0.78 MPa PEG solutions for germination improvement. 10 seeds of each differentially aged (G90% to G0%) in five replicates were placed for treatment. After one day priming at 30°C, the seeds were washed in sterile water for twice, blotted on filter paper and immediately used for germination tests and other analysis. The concentration of PEG solution was calculated according to Michel and Kaufmann [10].

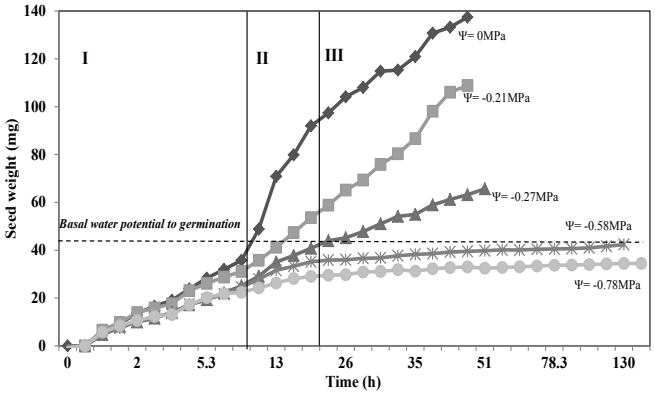

Figure 1: A tri-phase water uptake by the seed during germination. Phase I: initial rapid water uptake; Phase-II: lag phase with little net or no water uptake; phase-III: another rapid water uptake with rising up to critical moisture content and make radicle emergence and growth (Ψ=0 MPa, imbibition with water). During osmopriming, water uptake time prolonged depending on the concentration of osmoticum. Lower concentration of PEG (-0.21 MPa and -0.27 MPa) extends the phase-II and delays the germination, whereas high concentration (-0.58 MPa and -0.78 MPa) arrests in phase-II and prohibits phase-III. For the water uptake mean of seed weight with imbibition time taken; n=10 seeds used for individual event.

Seed germination and viability test

Germination was evaluated by measuring germination percentage obtained after 7 days. Germination trails were carried out in a dark room at 27-32°C temperature. Seeds were surface-sterilized (5 min, 1.0% sodium hypochlorite) and were placed on moistened filter papers with 2 ml sterile water in 12 cm diameter petri dishes [2]. The germination index was computed daily for seven consecutive days till radicle protrusion of at least 5 mm. Germination index/germination vigour is progressive total of daily cumulative germination over the time and expressed as GI=MDG × PV; where MDG: mean daily germination, which is the mean germination percentage per day and PV: Peak value of germination represent the maximum cumulative germination percentage divided by the number of days to reach this percentage [12]. Seed viability was evaluated by tri-phenyl tetrazolium chloride (TTC) reduction assay, which differentiates live from dead seeds based on activity of respiratory enzymes. Aged and their osmoprimed seeds were soaked in sterile water for 12 h and then placed in 0.5% TTC solution for 8 h (30°C) in the dark. The reduced TTC was extracted with ethanol and assayed spectrophotometrically at 520 nm [13].

Membrane integrity

Electrolyte leakage was determined as loss of membrane integrity; by placing 10 seeds into 5 ml of sterile water at 27-32°C in dark and conductivity of the medium was measured with a conductivity metre (Elico) after 24 h [12]. This experiment was conducted in five replicates. Results were expressed in mS/seed that represented the means of leakage measurements ± standard error (SE).

Lipid peroxidation

The occurrence of malondialdehyde (MDA), end product of the oxidation of polyunsaturated fatty acids, is considered a useful index of general lipid peroxidation. A common method for measuring MDA, referred to as the thiobarbituric acid-reactive substances (TBARS) assay, from Heath and Packer [14] and as modified by Du and Bramlage [15] to eliminate sugar interferences. Weighed amount of seed samples (500

mg) were homogenized at 26 ± 2°C with 0.5% (w/v) 2-thiobarbituric acid (TBA) dissolved in 20% (w/v) trichloroacetic acid (TCA). 1% (w/v) Butylated hydroxy toluene (BHT) was included in the reaction mixture to eliminate artifactual peroxidative damage to the samples during processing. The sample homogenates were heated in a 95°C water bath for 30 min, followed by 15 min on ice to remove the proteins and centrifuged (4°C) at 10000 g for 5 min. The clear supernatants were collected and the amount of malondialdehyde (MDA)-thiobarbituric acid (TBA) complex in the supernatant was measured by absorbance at 540 nm (TBARS products) and corrected for the non-specific absorbance by subtracting the value obtained at 600 nm (turbidity). Interfering absorbance were removed by recording absorbance at 440 nm (sugars) to eliminate the interference by sucrose. The amount of MDA was calculated with the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed in µmol MDA g^{-1} FW and corresponded to means of measurements carried out with four extracts ± SE.

MDA g^{-1} FW = {[A_{532} – A_{600}] – [A_{440} – A 600] × 0.057}/157000 × 10^6

Statistical analysis

Data were analyzed by one-way and three-way ANOVA in combination with the Duncan's multiple-range tests at 5% level of significance ($p \leq 0.05$) for post-hoc comparisons of means. The bars/lines having similar alphabets were statistically non-significant at $p<0.05$ level, according to Duncan's multiple-range tests. Statistical tests were carried out using SPSS (version-16) for Microsoft Windows. Data given in percentage were subjected to arcsine transformation before analysis.

Results

Seed viability represents a trait that is important for the conservation of seed resources. To test viability of Neem (*Azadirachta indica)* seeds need to be stored for a long time and assessed by germination ability. Post-harvest matured Neem seeds displayed high moisture content (0.53 ± 0.017 g H_2O g^{-1} DM) with 100% germinability. The moisture content rapidly declined up to 0.23 ± 0.023 g H_2O g^{-1} DM within 14 days after harvesting and thereafter gradually decreased under natural storage condition (Figure 2). Seeds exhibited 100% germination up to 33 days of storage.

Figure 2 demonstrates natural ageing (NA) characterized by statistically significant (<0.001) decline in germination percentage during storage. Neem seeds lost total viability within 129 days. The controlled deterioration test, defined as accelerated aging (AA), has been developed as an alternative to analyze this property more efficiently. AA conditions are utilized to speed up the loss of viability. We found that treatment at 45°C and 100% humidity could artificially accelerate the aging of Neem seeds (Figure 3). Loss of viability showed linear positive correlation with accelerated ageing (R^2=0.994). AA treatment for 15 h exhibited total loss of germination. Evaluation of seed viability by TTC reduction assay revealed that viable seeds showed by red stained. Viability gradually decreased in axes, however drastically reduction observed in cotyledon of NA and AA seeds (Figure 4). We observed that 12-17% improvement takes place in germination of differentially NA and AA aged seeds after priming treatment (Table 1). The germination improvement is limited at 50% germination level of ageing, below 50% failed. Accordingly, priming exhibited germination index (seed vigour) improvement and exhibited rapid germination (Figure 5). Loss

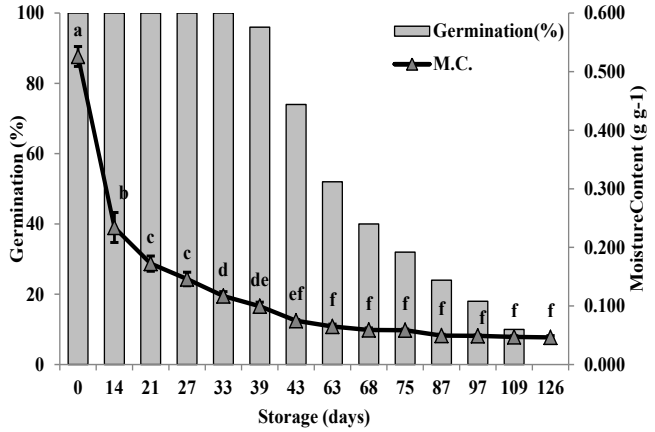

Figure 2: Effect of natural ageing (27 ± 1°C, RH 45-50%) on seed germination and moisture content. Each value is mean of five replicates ± SE. Data points showing the same letter are not significantly different at p<0.001.

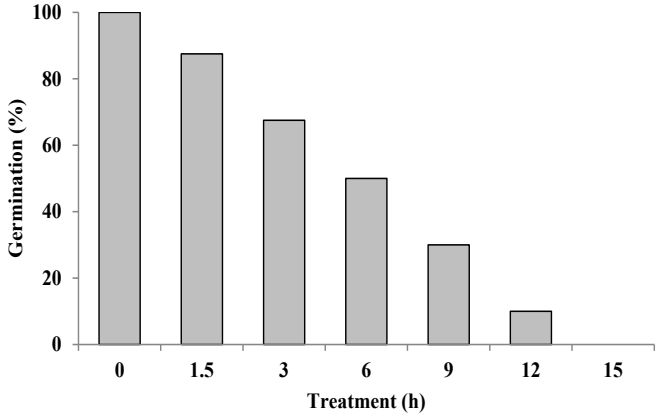

Figure 3: Effect of accelerated ageing (45°C, RH 100%) on seed germination. Seeds were treated at different time intervals. Decreasing germination percentage shows linear positive correlation with accelerated ageing treatment (R^2=0.994).

	Natural Aged	Primed	Improvement	Accelerated Aged	Primed	Improvement
$G_{90\%}$	96	100	4*	88	100	12*
$G_{70\%}$	74	86	12*	68	85	17*
$G_{50\%}$	52	66	14*	50	62	12 *
$G_{30\%}$	30	28	-2	30	30	0
$G_{10\%}$	10	13	3	10	10	0
$G_{0\%}$	0	0	0	0	0	0

* p<0.005 Significant Improvement

Table 1: Germination improvement by osmopriming treatment of differential aged Neem seeds at different levels of germination.

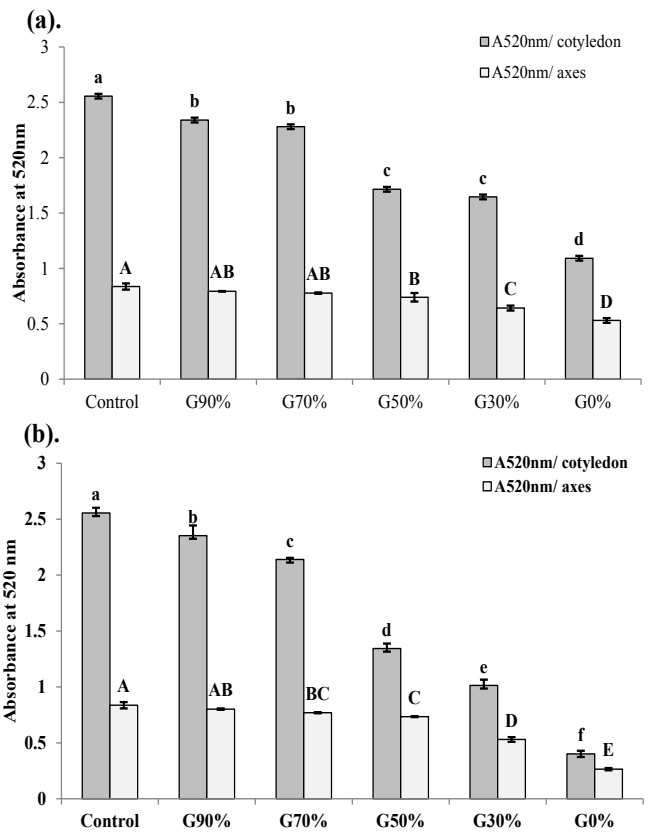

Figure 4: Effect of (a) natural and (b) accelerated ageing on Neem seed cotyledon and axes viability (as Reduced TTC content) at different germination percent. Vertical bars represent mean of independent replicates ± SE. Data points showing the same letter are not significantly different at p<0.001.

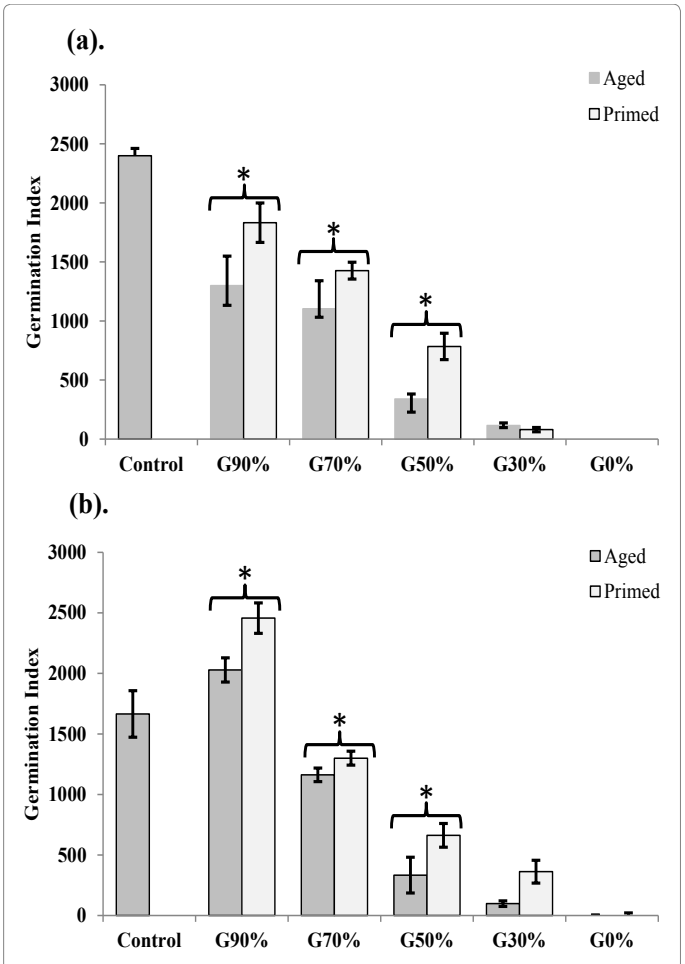

Figure 5: Effect of osmopriming on germination index (GI) of (a) natural and (b) accelerated aged Neem seed at different germination percent. Vertical bar represent mean of five independent replicates ± SE. Asterisk shows the significant (p<0.005) improvement in germination percentage by osmopriming.

of membrane integrity was exponentially increased in both NA and AA, but in comparison with the membrane permeability values NA was found to be superior to AA. Osmopriming treatment significantly improved the membrane damage at different levels of germination (Figure 6). Lipid peroxidation was evaluated by determination of seed malondialdehyde content. MDA content was around 1.52 ± 0.01 µmol/g FW in fresh seeds and increased gradually with ageing (Figure 7). Osmopriming showed a significant decline in MDA content compared to same aged seed, moreover reduction of MDA content by priming decreased with ageing.

Discussion

The phenomenon of ageing induces progressive deterioration in seeds leading to lethal damage and failure of germination. The most obvious way to test physiological basis of seed longevity is by storing seed under favorable conditions. Thereafter, the viability has to be tasted at regular intervals. The germination percentage and seed viability have to be correlated with the physiological property of seeds. During storage of seeds, germination capacity and seed vigour may be affected by ageing. Loss of germination capacity depends on seed whether orthodox, intermediate or non-orthodox and storage condition. Hence, seeds of many species can be stored only for months, while other species remain viable for several decades or more [16-18]. Seeds viability depends upon two factors: temperature and moisture content. Under constant temperature (26°-28°C and RH 45-50%) Neem (*Azadirachta indica)* seeds show loss of their moisture content and viability during storage (Figure 2). The results show that seed viability is strongly associated with their moisture content. Below the lowest safe moisture content (0.12 ± 0.008 g H_2O g^{-1} DM) seeds start losing their viability. Moreover in natural aging, there are some valuable studies on biochemical and physiological deterioration during seed aging under accelerated aging conditions using high temperature and high seed moisture content [19,20]. Depending on their physiological structures, seeds typically lose their viability within a couple of days or weeks under such storage conditions. Controlled deterioration (45°C, RH 100%) with Neem seeds showed a linear loss of viability with accelerated ageing treatment (Figure 3). With differential natural and accelerated ageing, two types of survival (germination percentage) curves were obtained. Long term storage (NA) has an initial period of no or slow mortality followed a sigmoidal type but in controlled deterioration (AA), there is no evidence of initial relative storage stability. Such survival curves concluded that temperature and/or moisture content should be the expected cause of transition from relative stability to sigmoid deterioration within the same seed lot. Tetrazolium chloride test, a rapid method of estimating seed viability revealed that seeds endosperm tissue is drastically affected as compared to seed axes during ageing (Figure 4). It is believed that such tissue viability is affected with their moisture content [21]. Seed priming (pre-sowing imbibition

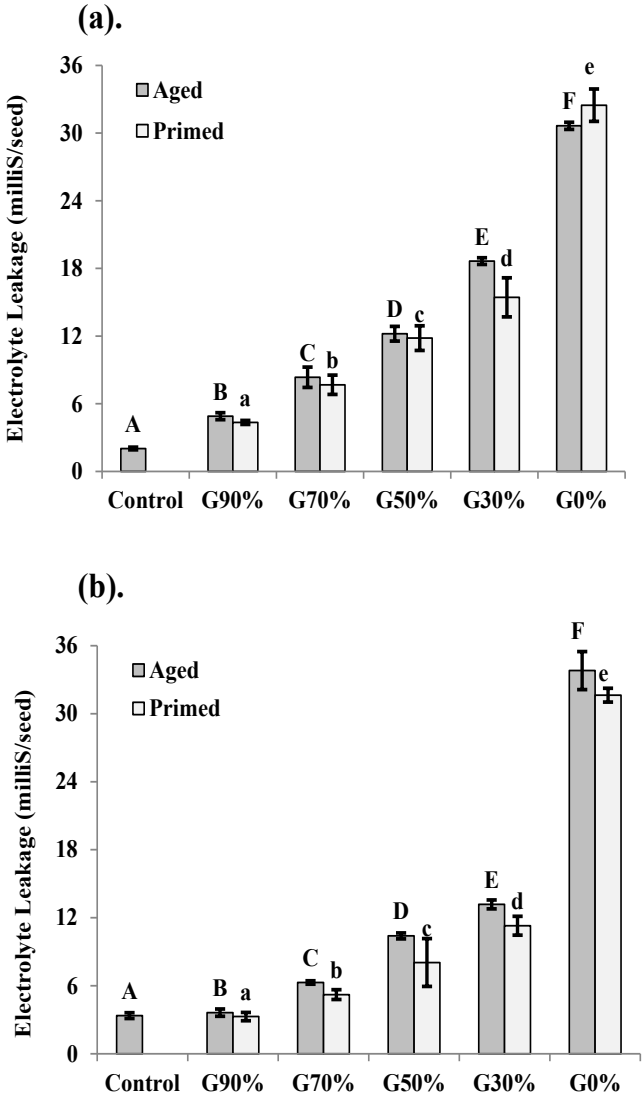

Figure 6: Effect of osmopriming on electrolyte leakage of (a) natural and (b) accelerated aged Neem seed at different germination percent. Vertical bar represent mean of five independent replicates ± SE. Membrane damage exponentially increased with time of ageing and improved with osmopriming.

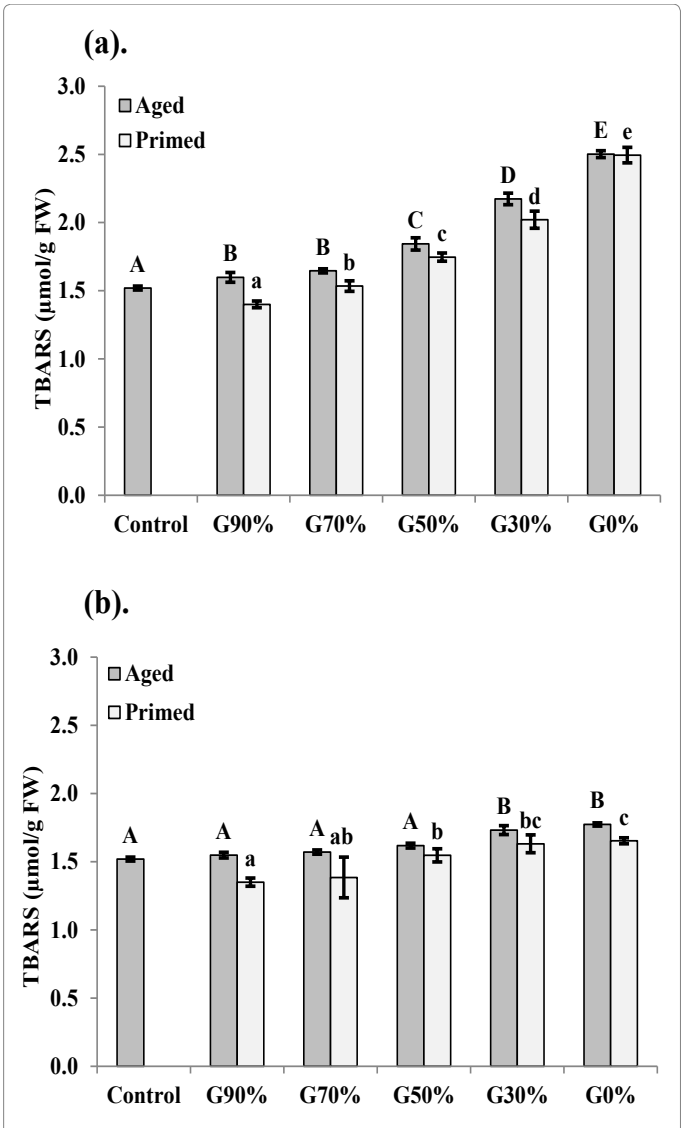

Figure 7: Effect of osmopriming on Lipid peroxidation (TBARS) of (a) natural and (b) accelerated aged Neem seed at different germination percent. Vertical bar represent mean of five independent replicates ± SE.

treatment) is widely used to enhance seed performance with respect to rate and uniformity of germination [3,22,23]. There are many factors associated with the effects of seed priming, but the concentrations of priming solutions as well as the time and temperature during priming were crucial [24]. Osmopriming optimized with -0.78 MP PEG-6000 concentrations at 30°C for one day are the best with reference to germination response and germination index (Supplementary Table 1). The results of this study indicate that osmopriming of aged seeds has significant positive effect on the germination and seed vigour. Similar results, i.e., priming induced improvement in seed germination have been reported earlier [8,25-28]. However, our data indicates that 12-17% improvement occurs in primed seed compared to aged seeds; such improvement only limited up to 50% germination level of ageing (Table 1). No improvement occurs below 50% germination level of ageing. Seed vigour is a complex physiological feature that ensures rapid emergence of plants, but decreases with seed viability during ageing. Seed vigour improved following seed priming treatment (Figure 5). Membrane integrity was strongly related to loss of seed viability. Crowe et al. [29] suggested that biological membranes are particularly sensitive to dehydration. Hydrated bilayer structure of membrane generally remains in the liquid crystalline phase. Dehydration removes water from within the phospholipids of membrane and consequently lateral spacing between phospholipids is reduced. This leads to increase opportunities for van der Waals interaction between the acyl chains of phospholipids and consequently formation of gel phase. Neem seed has been categorized as having intermediate storage longevity [1,30,31]. Their limited desiccation tolerance has been particularly attributed to their sensitivity to imbibition stress. Membrane exhibited holes within seconds after imbibition when they were in gel phase just before imbibition. Hydrated seeds in bulk water it undergoes a phase of transition and are expected to leak. In the present work, we observed during imbibition (bulk water) a significant increase in electrical conductivity with both natural and accelerated ageing, and membrane

damage, evaluated by electrolyte leakage, was linearly associated to seed germination. As germination percentage decreased with ageing, electrolyte leakage exponentially increased (Figure 6). According to Chang and Sung [32], the ageing treatment causes inability of seeds to maintain the integrity of membranes leading to reduced germinability of seeds. During osmopriming (limited hydration), it passes through the phase transition that decreases leakage of solute attributed to better repair of membrane. Moreover, both types of ageing (NA and AA) show significant (<0.001) increased MDA content in seed with loss of viability, and it was mainly higher in seed kept under natural ageing (NA) storage compared to AA (Figure 7). Gradual increase of MDA content in each level of ageing from G100% to G0% observed in this study corroborate with reports published earlier [33,34]. The highest MDA content with 0% germination during AA was found after 15h of accelerate ageing treatment. This indicates that seed exposure to extreme conditions (40°C and relative humidity of 100%) may lead to highly pronounced peroxidative lipid degradation in seed that was equal to the observed findings in seed stored for 2 months with 50% germination under NA. This indicates that AA caused changes in seed that are not equal to the changes occurred in seed during NA [35,36]. Lipid peroxidation generates change in the structural and functional properties of membrane lipids, which increases membrane damage. In this study, although a positive correlation between membrane damage and lipid peroxidation was observed, but lipid peroxidation is not associated with viability loss. Osmopriming has strong positive correlation with germination during ageing (Supplementary Table 3), that improves germination, germination vigour, membrane integrity and lipid peroxidation level.

Conclusion

Loss of viability and germination associated with non-orthodox (intermediate and recalcitent) seed is well known. Germinating seed must undergo many cellular and metabolic changes during imbibition. In the past decade, lots of researches have been focused on seed priming for improvement of seed germination, but the limit of improvement is yet to be ascertained. The present findings further indicate that both natural and accelerated ageing represent similar pattern except their mortality curve. In natural ageing prior to entering sigmoidal type decline, a period of relative stability exists; whereas in accelerated aging, such relative stability is absent. Such survival curves are model of dynamic rate of mortality and highly influenced with moisture content and temperature. It is strongly established that the seed priming improves the seed germination until a critical level (boundary) during the ageing and thereafter priming does not support the process of improvement of germination.

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