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Kinetin-Mediated Prolongation of Viability in Recalcitrant Sal (*Shorea robusta* Gaertn. f.) Seeds at Low Temperature: Role of Kinetin in Delaying Membrane Deterioration during Desiccation-Induced Injury

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Abstract. The effects of kinetin (6-furfurylaminopurine) on viability during storage of recalcitrant sal (*Shorea robusta* Gaertn. f.) seeds at low temperature (15°C) were investigated. The freshly mature sal seeds showed an absolute loss of viability within 6–7 dah (days after harvest) when stored at ambient or at 15°C (control). Storage of these seeds at 15°C after kinetin (10 ppm) treatment prolonged the viability period up to 35 days with 20% germination. The kinetin-treated seeds exhibited 100% germination up to 10 days compared with 3 days in controls. Measurements of leachate conductivity, $\cdot O_2^-$ and lipid peroxidation registered gradual increases from 0 dah onward to 35 dah with significantly low levels compared with controls. On the other hand, an enormous increase in superoxide dismutase activity was discernible for a longer duration (0–35 dah) in kinetin-treated seeds than in control seeds where it remained for 3 dah. The role of kinetin in prolonging seed viability by reducing the loss of leachates, lipid peroxidation, $\cdot O_2^-$, and enhancing of superoxide dismutase is discussed.

Key Words. Seed viability—Oxidative stress—Extension—Kinetin recalcitrant–*Shorea robusta* (Sal)

Sal (*Shorea robusta* Gaertn. f.) makes up almost 14% of the total forest cover of India (Joshi 1980). It is valued for its timber and for the oil from its seeds. The seeds are short lived under natural conditions; storage and transport problems have hindered the establishment of plantation and species trials (Tompsett 1985). These problems indicate the importance of developing methods for extending the storage life of the seeds.

Like many other recalcitrant seeds, sal seeds do not show maturation drying. They are shed from the parent plant at high moisture content (42–49%). The recalcitrant nature of sal seeds excludes all traditional methods of storage. The rapid loss of moisture content and concomitant loss of germination, particularly below the relatively high LSMC (lowest safe moisture content) value, in almost all recalcitrant seeds, has posed a great challenge in their storage (Chin and Roberts 1980). Because the physiology of development and storage of recalcitrant seeds (Berjak et al. 1990) are altogether different from those of orthodox seeds, attempts have been made to develop new storage methods. The temperate-recalcitrant species, such as *Quercus* and *Aesculus*, have seeds that cannot be dried at all, but they can be stored for several years (1–3 years) at near freezing temperatures (3 to −3°C) with marginal loss in viability (18–35%) (Bonner and Vozzo 1987, Tylkowski 1984). On the other hand, the seeds of tropical recalcitrant species have the same high moisture requirement as the temperate-recalcitrant species, but they are sensitive to low temperature (Chin and Roberts 1980, Yap 1986). Even short periods of storage at chilling temperatures will cause rapid loss of viability. Included in this group are many *Shorea* species (Purohit et al. 1982, Yap 1986), *Theobroma cocoa* (King and Roberts 1979), *Hopea* species (Song et al. 1984), and several tropical forest tree seeds (Chin and Roberts 1980).

In general, the viability of most of the tropical recalcitrant seed species can be prolonged, for a limited period, when stored at low temperatures (13–15°C). For example, sal seed viability was extended over a period of

Abbreviations: LSMC, lowest safe moisture content; SOD, superoxide dismutase; RH, relative humidity; DW, distilled water; TBRS, thiobarbituric acid-reactive substance(s); MDA, malondialdehyde; FW, fresh weight; PVP, polyvinylpyrrolidone 40; dah, days after harvest.

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30–50 days (30–60% viability) (Tompsett 1985), 30 days (40% viability) (Purohit et al. 1982), 49 days (35% viability) (Khare et al. 1987), and 18 days (35% viability) (Tompsett 1985) at 13°C. The difference in the prolongation periods could be the result of either the source (provenance) from which the seeds were collected or the initial viability. A similar conclusion was drawn by Ellis et al. (1990) in regard to the sensitivity of seed lots from different provenances to damage due to chilling and desiccation. Further, in extension experiments, no record of the percent of moisture content at low temperatures was made, which is vital in desiccation-sensitive recalcitrant seeds, with the exception of the study by Tompsett (1985), who reported 41% moisture content on the 18th day.

The freshly mature sal seeds (Chaitanya and Naithani 1994) collected from the Raipur and Bastar districts (among the richest sal forests in India) showed negligible extension of viability when stored at 15 ± 2°C (unpublished). Hence, an attempt has been made to prolong the viability by using phytohormones. Saha and Takahashi (1986) were the first to speculate on the role of auxin and kinetin in prolonging the viability of *S. robusta.* Phytohormones, particularly cytokinins, have been considered the most effective hormones in delaying senescence (Nooden 1980). The basic biochemical phenomena operating during senescence and/or seed aging appear to be similar at the cellular level. Therefore, in the present study an attempt was made to assess the capacity of cytokinin to prolong the viability in sal seeds. We have reported previously that membranes are the key sites of injury in naturally aging sal seeds (Chaitanya and Naithani 1994). Enhanced leakage, $\cdot O_2^-$, and lipid peroxidation were shown to be closely related to desiccation-induced deterioration in the sal seeds. The role of cytokinin in scavenging $\cdot O_2^-$ (Lesham 1987) and maintaining high activity of scavengers is well established. Therefore, in the present study efforts were also made to assess the biochemical basis of prolonging seed viability by monitoring the activity of superoxide and its scavenging enzyme superoxide dismutase (SOD) during seed aging.

Materials and Methods

Collection of Seed

In this study, the seeds were collected from Gariyabandh Forest (90 km away from Raipur). The forest is situated to the northeast of Raipur and lies between 20°38′N latitude and 82°04′E longitude. Its elevation is 306 meters above sea level. Nearly 25–30 trees in the forest were marked for collection of fruits and seeds. Fully mature seeds (showing 100% germination within 40–48 h) of sal 63 days after anthesis were collected and brought to the laboratory within 4–5 h (Chaitanya and Naithani 1994).

Pretreatment of the Seeds and Their Storage

The calyces of the seeds were plucked manually, and the healthy and uninfected seeds of uniform size were sorted out. About 8,000 seeds from the freshly harvested seed lot were divided into four lots of 2,000 seeds each. Nearly 50 seeds from the lot were separated, and their initial moisture content was determined. Three seed lots were pretreated with 5, 10, and 20 ppm kinetin (6-furfurylaminopurine; Sigma) for 8 h. Simultaneously, the fourth seed lot was treated with distilled water for 8 h and used as a control. All of these seed lots were then dried to their initial moisture content and stored in separate trays at 15°C ± 2°C and 40–45% relative humidity (RH) in a biological incubator (Sico, India). Seeds were harvested regularly to carry out various analyses. The biochemical analyses were performed on the embryonic axes, which are the location of the major changes during the loss of viability (Bewley 1986).

Germination

Seeds were surface sterilized with $HgCl_2$ (0.1%) washed thoroughly four or five times with distilled water (DW), allowed to imbibe DW for 24 h, and germinated on water-saturated filter paper in Petri dishes. Germination was scored every 24 h as radical emergence to 5–7 mm. Linear regression of probit percentage germination against moisture content percentage was performed per Finney (1971).

Moisture Content

The moisture content (percent fresh weight basis) of the seeds was determined using the formula given by the International Seed Testing Association (1985).

Leakage Loss

The leachates were collected after 24 h of imbibition of water by the seeds and estimated by measuring the specific conductivity using a conductivity meter (Systronics).

Lipid Peroxidation

Lipid peroxidation was measured as the concentration of thiobarbituric acid-reactive substances (TBRS), equated with malondialdehyde (MDA) (Heath and Packer 1968), and expressed as A_{540}/g FW of the sample as described elsewhere (Chaitanya and Naithani 1994).

Superoxide Determination

Weighed amounts of embryonic axes were homogenized in cold (0–4°C) sodium phosphate buffer, (0.2 M, pH 7.2) containing diethyl dithiocarbamate (10^{-3} M) to inhibit SOD activity. The homogenate was centrifuged immediately for 1 min at 5,367 ×*g*. In the supernatant, the superoxide anion ($\cdot O_2^-$) was measured by its capacity to reduce nitro blue tetrazolium (2.5×10^{-4} M). The absorbance of the end product was measured at 540 nm. $\cdot O_2^-$ formation was expressed as ΔA_{540}/min/g FW of the sample.

SOD Activity

Weighed amounts of embryonic axes were homogenized in ice-cold 0.2 M borate buffer, pH 7.4 (sodium tetraborate plus boric acid) containing

Table 1. Dose response of different kinetin concentrations on the viability of sal seeds.

Days after harvest	% Germination			
	Control $(DW^a)_{15°C}$	Kinetin		
		5 ppm	10 ppm	20 ppm
0	100	100	100	100
1	100			
2	100			
3	100			
4	100			
5	60 ± 5.0	80 ± 5.0	100	100
6	40			
7	20			
8	0			
10	0		100	
15	0	40 ± 5.0	80 ± 5.0	50 ± 5.0
25	0	0	40	20 ± 3.53
35	0	0	20	0

[a] DW, distilled water.

25% PVP (polyvinylpyrrolidone 40). The homogenate was centrifuged at 17,890 ×*g* for 10 min. The supernatant was subjected to acetone precipitation at 0–4°C (Naithani 1987) and again centrifuged at 5,367 ×*g* for 3 min. The pellet was resuspended in sodium phosphate buffer (0.02 M, pH 6.4) and used as an enzyme source. SOD activity was determined by measuring the inhibition of pyrogallol autoxidation at 420 nm and quantified by the method of Marklund and Marklund (1974). SOD activity was expressed as units of SOD min^{-1} g^{-1} FW of the sample (embryonic axes). All spectrophotometric analyses were carried out using a 160-A UV-visible spectrophotometer (Shimadzu).

Results

Dose Response: Percent Germination

The germination response of the sal seeds to various doses of kinetin treatment (5, 10, 20 ppm) was evaluated (Table 1). Of all of the treatments, the maximum response was observed in seeds treated with 10 ppm kinetin; that is, the germination was extended up to 35 days after harvest (dah) (with 20% germination). Even 100% germination was observed up to 10 dah in kinetin-treated seeds compared with 4 dah in control seeds stored at 15°C. Although the 5 ppm and 20 ppm kinetin-treated seeds also exhibited germination on 15 dah, it was only 40 and 50%, respectively. In the 5 ppm kinetin-treated seeds, the percent germination declined from 100 to 80% by 5 dah and to 40% on 15 dah. In the 20 ppm kinetin-treated seeds, 100% germination was recorded up to 5 dah. The percent germination declined to 50% by 15 dah. Hence, 10 ppm kinetin was chosen for further experiments.

Moisture Content

A rapid decline in the percent of moisture content (Fig. 1) in the control seeds was discernible from 0 to 10 dah (from 42.2 to 16.7%, respectively), whereas kinetin treatment resulted in a delay in the desiccation of the sal seeds. The kinetin-treated seeds exhibited a gradual decline in the percent moisture content from 0 (42%) to 35 dah (19.2%) (Fig. 1), with LSMC 37% (Fig. 2). The percent moisture content was registered to be 34% on 15 dah, when percent germination declined to 80%. Later on, by 25 dah, the percent moisture content declined to 28.6% and to 19% on 35 dah, at the end of the study. In the kinetin-treated seeds, a linear relationship was established between the probit viability and the percent moisture content, expressed by the regression line $Y = -58.0548 + 3.832x$. A close correlation was established between probit viability of kinetin-treated seeds and their percent moisture contents ($r = 0.97$; $p < 0.001$) (Fig. 2).

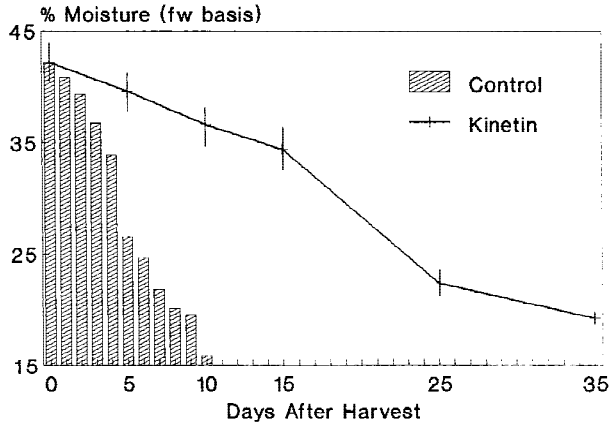

Fig. 1. Decline in percent moisture content with age in control and kinetin-treated sal seeds at 15°C. Each value is the mean of 50 observations. *Vertical bars* represent ± S.D.

Leakage Loss

Kinetin treatment not only delayed the leakage from the sal seeds with aging but also reduced the extent of leakage (Fig. 3) during imbibition. Control seeds recorded a 10-fold increase in leachate conductivity in 10 days, whereas the kinetin-treated seeds exhibited a 6-fold increase in 35 days. Kinetin treatment reduced the leakage loss by more than 20%. The maximum specific conductivity of the leachates from kinetin-treated seeds was observed to be 0.73 mMhos on 35 dah. By 10 dah, when the leachate conductivity of the control seeds was 0.96 mMhos, the leachate conductivity of kinetin-treated seeds was only one fourth of that in control seeds, 0.23 mMhos.

Lipid Peroxidation

Lipid peroxidation/accumulation of TBRS in the kinetin-treated seeds exhibited a trend similar to that in the control seeds (Fig. 4). The accumulation of TBRS in the

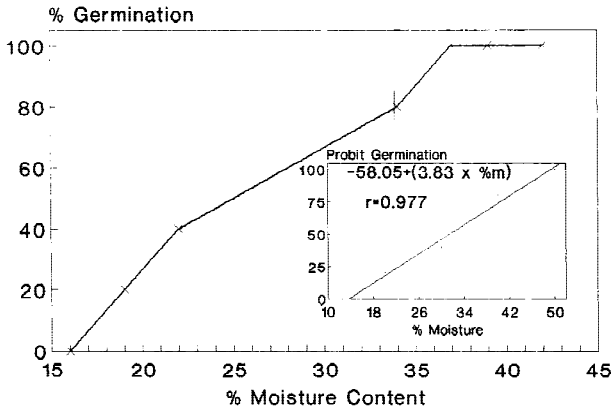

Fig. 2. Decline in percent germination with lowering moisture content in kinetin-treated sal seeds. Each value is the mean of 50 observations. *Vertical bars* represent ± S.D. *Inset*, positive correlation between probit germination and percent moisture ($r = 0.97$, $p < 0.001$).

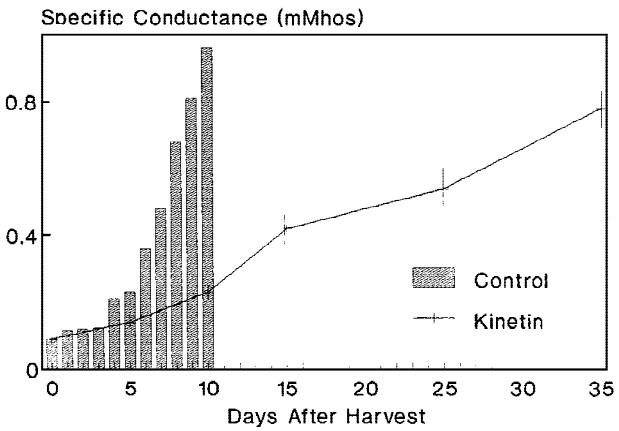

Fig. 3. Loss of electrolytes from kinetin-treated and control sal seeds. Each value is the mean of 50 observations. *Vertical bars* represent ± S.D.

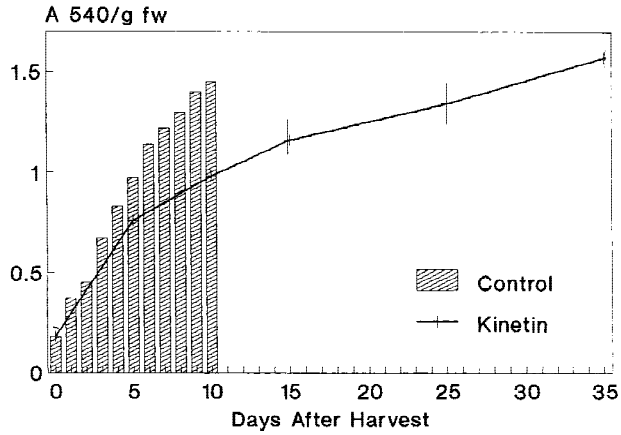

Fig. 4. Changes in lipid peroxidation activity in the embryonic axes of control and kinetin-treated sal seeds. Each value is the mean of six observations. *Vertical bars* represent ± S.D.

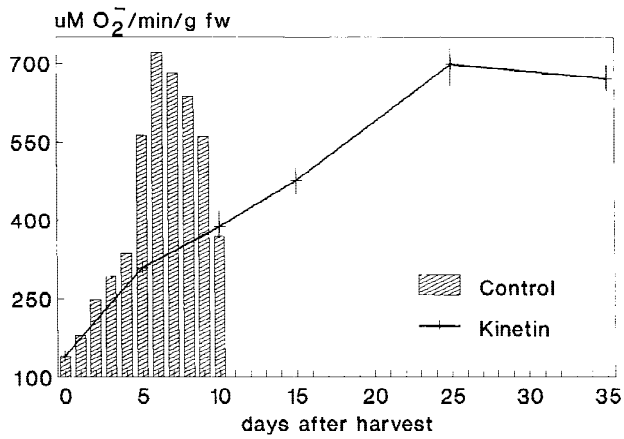

Fig. 5. Changes in the level of superoxide radicals in the embryonic axes of control and kinetin-treated sal seeds. Each value is the mean of six observations. *Vertical bars* represent ± S.D.

control seeds was rapid and high, from 0.21/g FW (0 dah) to 1.45/g FW (10 dah), whereas the accumulation of TBRS in kinetin-treated seeds was relatively slow; on 10 dah, it was only 0.8/g FW (1.5 times less than that in control seeds). The magnitude of TBRS in kinetin-treated seeds on 35 dah was equivalent to that of the control on 10 dah.

Superoxide Liberation

Kinetin not only reduced the $\cdot O_2^-$ level but also delayed (up to 15 dah in treated seeds) the accumulation of $\cdot O_2^-$ compared with 7 dah in control seeds (Fig. 5). In the control seeds, a sixfold increase was recorded within 7 dah, whereas the kinetin-treated seeds showed only a twofold increase in the same period. The liberation of superoxide radicals in the kinetin-treated seeds was relatively gradual and not as abrupt as in the control seeds.

SOD Activity

Kinetin not only enhanced the levels of SOD but also maintained higher SOD activity for a longer duration (Fig. 6). The kinetin-treated seeds registered peak activity on 10 dah, with almost a 1.5-fold increase compared with the control on 3 dah. The pattern of changes in the SOD activity was, however, similar in both control and kinetin-treated seeds. A sharp increase in SOD activity (maximum activity of 1.15 units/mg of protein on 10 dah) was followed by a sharp decline on 15 dah (0.593

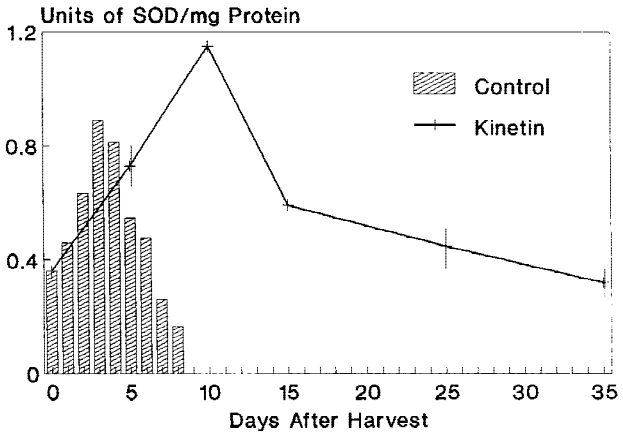

Fig. 6. Changes in SOD activity in the embryonic axes of control and kinetin-treated sal seeds. Each value is the mean of six observations. *Vertical bars* represent ± S.D.

units/mg of protein), and thereafter the decline was gradual up to 35 dah (0.32 units/mg of protein).

Discussion

Freshly harvested sal seeds, with 42% moisture when stored at ambient conditions (Chaitanya and Naithani 1994) and 15°C (Table 1), lose viability after 4 dah. Pretreatment of seeds with kinetin averted the loss of germinability when seeds were stored at 15°C. However, no improvement was recorded when the kinetin-treated seeds were kept at ambient temperature (unpublished). In kinetin-treated sal seeds, 100% viability was recorded up to 10 dah; and even at 35 dah, the germination was registered to be 20%. Because the moisture content in desiccation-sensitive sal seeds is closely associated with germination (Chaitanya and Naithani 1994), the retention of a high moisture content (LSMC 37%) up to 10 days in kinetin-treated seeds, for a longer duration, is of utmost importance. For example, the nonviable untreated sal seeds retained only 17% moisture content on 10 dah when stored at 15°C, whereas a relatively very high moisture content, near 37%, was retained in kinetin-treated seeds of same age. Even on 35 dah, the moisture content was around 20% and so was the percent germination (20%). It appears that kinetin treatment offers a means of retaining high moisture for longer period in sal seeds, thus prolonging their viability. Furthermore, the linear relationship between probit viability and loss of moisture, which is an important feature of all desiccation-sensitive/recalcitrant seeds (Berjak et al. 1990, Finch-Savage 1992, Pritchard 1991, Probert and Longley 1989) has been observed to hold true even in the kinetin-treated sal seeds (Fig. 2).

A variety of physiologic and biochemical changes has been noted during storage at 15°C in the kinetin-treated sal seeds. The proposal that decreased membrane integrity and the occurrence of membrane lesions might play a significant role in the deterioration of seeds (Harman and Mattick 1976, McKersie and Stinson 1980, Senaratna and McKersie 1983) has been supported by recording enhanced solute leaching accompanying a fall in germinability/viability in other seeds (Bewley 1986, Delouche 1969, Parrish and Leopold 1978, Roberts 1979) and in deteriorating sal seeds (Chaitanya and Naithani 1994, Nautiyal and Purohit 1985, Yadav et al. 1987). The data reported in Fig. 3 confirm that the desiccation-induced loss of sal seed viability may be caused by membrane perturbations as a rapid increase in leachate conductivity was recorded in untreated sal seeds. Substantial suppression of this deleterious effect (reduced levels of leachates up to 35 dah) as a result of seed pretreatment with kinetin suggests that kinetin may have induced tolerance to desiccation-sensitive seeds by retaining the membrane integrity.

Several reports (Dey and Mukherjee 1988, Simon 1974, Wilson and McDonald 1986) have concluded that a close correlation exists between membrane permeability and lipid peroxidation. Stewart and Bewley (1980) have shown an increase in the leakage of metabolites from aged soybean axes with lipid peroxidation. In kinetin-treated sal seeds, lipid peroxidation was observed to occur more slowly than in untreated seeds (Fig. 4). In addition, kinetin-treated seeds also exhibited lower levels of lipid-peroxidized products up to 25 dah. Theories on the mechanism of lipid peroxidation suggest that fatty acids having two or more unsaturated bonds are prone to free radical attack (Funes and Karel 1981, Gutteridge and Halliwell 1990, Pauls and Thompson 1981, Roubal 1970). In the present study, measurements of oxygen free radicals in control and kinetin-treated seeds showed significantly higher levels and rates in untreated control seeds (Fig. 5). Free radical-mediated damage to macromolecules, as well as membrane lipids in seeds, is proposed (Desai and Tappel 1965, Halliwell and Gutteridge 1984, Pan and Yau 1991). Harman and Mattick (1976) have suggested that free radicals might interact with the membrane phospholipids and lead to their deesterification, thus resulting in the accumulation of fatty acids and increased membrane dysfunction (Hoekestra et al. 1989, Senaratna et al. 1984, 1985). Loss of desiccation tolerance during germination was accompanied by a fourfold increase in the accumulation of MDA, a measure of peroxidative damage to lipid acyl chains (Leprince et al. 1990). Our data therefore indicate that catabolic processes, like superoxide-mediated lipid peroxidation, were reduced drastically in kinetin-treated seeds. Although the levels of lipid peroxidation and $\cdot O_2^-$ increased throughout the analysis period even in the kinetin-pretreated seeds, these changes were much more pronounced in untreated seeds. Thus, it is suggested that

kinetin is effective in maintaining seed viability, at least up to 35 dah, by reducing the accumulation of superoxides possibly by reducing the liberation of O_2^- radical, by scavenging the radical (Lesham 1987) or by maintaining higher levels of scavenging enzymes, thus reducing the series of consequences resulting from increased oxidative stress by $\cdot O_2^-$ in sal seeds.

Significantly higher levels of SOD were recorded in kinetin-treated seeds than in untreated seeds of sal. It is apparent that substantially higher levels of SOD in kinetin-treated sal seeds for extended duration could be the cause of correspondingly reduced levels of $\cdot O_2^-$ in these seeds, finally leading to the suppression of the chain of events mediated by $\cdot O_2^-$: lipid peroxidation, leakage loss, and increased membrane permeability. Enhanced levels of SOD caused by synthesis (Bowler et al. 1989, Casano et al. 1994) or activation of preexisting inactive SOD have been discussed amply in relation to tolerance during water stress, desiccation, etc. Highly reduced levels of SOD for a short span (up to 3 dah) in naturally aging sal seeds have been correlated with a rapid loss of viability (Chaitanya and Naithani 1994).

Considering the physiologic and biochemical changes analyzed in the kinetin-treated sal seeds, it can be concluded that kinetin inhibits/reduces the damage to membranes by effectively suppressing oxidative stress due to superoxide-mediated lipid peroxidation of membrane lipids by enhancing SOD activity for extended periods. These kinetin-induced changes finally lead to decreased peroxidation of phospholipid moieties, perhaps making these hydrophilic sites available for binding water molecules and causing the retention of a higher moisture content. Restoration of these phospholipid groups by lower rates of lipid peroxidation in kinetin-treated sal seeds appears to be the plausible cause for maintaining higher moisture content associated with desiccation tolerance and prolonged viability. From these investigations, kinetin appears to be a promising growth regulator to enhance the storage potential of true recalcitrant seeds in general and sal seeds in particular. However, the exact mechanism by which the kinetin regulates the suppression of desiccation-induced sequential deteriorative biochemical/cellular changes in prolonging viability of recalcitrant sal seeds needs further investigation.

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