Indian J. Plant Physiol., Vol. 15, No. 2, (N.S.) pp. 192-197 (April-June, 2010)

SHORT COMMUNICATION

CHILLING INDUCED SUPEROXIDE PRODUCTION, LIPID PEROXIDATION AND LEAKAGE LOSS IN *SHOREA ROBUSTA* SEEDLINGS

S. KESHAVKANT* AND S.C. NAITHANI

School of Life Sciences, Pt. Ravishankar Shukla University, Raipur-492 010, India

Received on 8 Dec., 2009, Revised on 14 May, 2010

Aerial parts of the chilling sensitive young sal (*Shorea robusta*) seedlings showed excess generation of active oxygen species (AOS) and malondialdehyde (MDA) in response to exposure of chilling temperature (9-14.1°C) during November to March in field conditions. Approximately 5-6 fold increase in AOS was estimated in aerial parts of chilling exposed seedlings than the control (greenhouse) seedlings. Accumulation of AOS was found to be closely associated with the rise in MDA in leaf (4.4 fold) and shoot (3.8 fold) tissues, whereas, control seedlings exhibited insignificant accumulation of both. Chilling exposed seedlings also showed significant promotion in the leakage loss of organic (protein) and inorganic ($K^+$and Ca^{++}) electrolytes from leaf and shoot tissues, but an insignificant leakage of electrolytes was recorded in greenhouse seedlings. The field grown sal seedlings (exposed to chilling) revealed a strong positive correlation between the rates of AOS and MDA accumulation (r = 0.98) and also between magnitude of MDA levels and leakage loss of various electrolytes (r = 0.99**), during chilling periods. Our results clearly showed that leaf and shoot of field grown sal seedlings are severely damaged due to chilling stress, whereas, the protected (greenhouse) seedlings are showing vigorous growth during same periods of analysis.**

Key words: **Chilling injury, leakage loss, malondialdehyde, *Shorea robusta*, superoxide.**

www.IndianJournals.com
Members Copy, Not for Commercial Sale
Downloaded From IP - 44.213.60.33 on dated 23-Jul-2024

Tropical and sub-tropical plants exhibit marked physiological and biochemical dysfunctions commonly referred to as chilling injury, when they are exposed to temperatures below 0-15°C (Saltveit 2002). These modifications include reduced germination and seedling development, tissue chlorosis and necrosis, increased electrolyte leakage, unregulated metabolism and accelerated senescence (Lafuente *et al*. 2004), all of which may impair optimum growth, development and quality of plants. Under stressful conditions like chilling, plant cells accumulate toxic by-products like active oxygen species (AOS) (Apostolova *et al*. 2008). Excess accumulation of these AOS can cause damage to cellular components severely disrupting metabolic functions (Suzuki and Mittler 2006). AOS can react with poly unsaturated fatty acids to cause peroxidation of essential membrane lipids in plasmalemma which leads leakage of cellular contents, rapid desiccation and cell death (Karabal *et al*. 2003). At chilling temperature, the membrane lipids solidify that causes cracks or channels leading to increased permeability (Lyons 1973, Campos *et al*. 2003). Temperature dependent phase transition of membrane lipids has been postulated to be the primary molecular event in plant species sensitive to chilling temperatures (Uemura *et al*. 2003) that resulted in loss of membrane integrity, as shown by severe electrolyte leakage and loss of osmotic responsiveness (Kirakosyan *et al*. 2003, Campos *et al*. 2003).

*Corresponding author, E-mail: skeshavkant@gmail.com

www.IndianJournals.com
Members Copy, Not for Commercial Sale
Downloaded From IP - 44.213.60.33 on dated 23-Jul-2024

Chilling stress in plants resulted in severe leakage loss of inorganic (K^+, Ca^{++}) and organic (soluble sugars, amino acids and proteins) electrolytes (Chang *et al*. 2001) that are vital for overall growth and development of plants. Proteins are required for acquisition of chilling tolerance in plants (Uemura *et al*. 2003) because of their water binding properties (Ismail *et al*. 1999). Similarly K^+, the dominant cation in the mature plant cells, is distributed in the vacuole and cytoplasm (Leigh and WynJones 1984). It has a purely osmotic role in the vacuole, whereas in the cytoplasm, K^+ has biochemical role including cofactor of several enzymes (Evans and Sorger 1966). Ca^{++} plays important role in the structure and function of membrane (Cheour *et al*. 1991) and cell wall (Glenn *et al.* 1988) including its central role in the cell metabolism (Cheour *et al*. 1991) and delay in leaf senescence (Pooviah and Leopold 1973).

Recently we have reported chilling injury as a major cause of mass (up to 80%) mortality in sal seedlings (Keshavkant and Naithani 2007). Seedling mortality in field as well as in nursery is a foremost drawback in regeneration of sal forest (Sinha 1956). The seedlings were shown to exhibit severe reduction in growth followed by permanent damage to aerial parts from November to March in response to constant chilling exposure (9-14.1^{o}C). In contrast, the seedlings protected from chilling temperature (in greenhouse) showed vigorous growth during November to March. Hence, to understand further the chilling injury superoxide generation, MDA production and leakage loss were investigated in sal seedlings exposed (field grown) and non-exposed (greenhouse maintained) to chilling temperature.

Mature sal seeds were collected from the Gariyabandh forest reserve, 91 km to North East of Raipur (20^{o}38'N latitude, 82^{o}04'E longitude and 306 masl). The seeds were germinated, immediately after collection, between two wet jute bags. On emergence of radicle (10-15 mm) after 2-3 days, the germinants were sown in polybags (15x22 cm) containing mixture of black soil, sand and farm yard manure (2:1:1, V:V:V). The seedlings were watered twice a day. The first sampling was made immediately after 7 days of emergence of first two leaves. Subsequently seedlings were sampled after every 30 days, from the day of emergence of the first two leaves. Four months after seedlings emergence, i.e. in October, the seedlings were randomly divided into two groups of 4000 seedlings each, of which one group was shifted to greenhouse (Temperature 30-32^{o}C, Relative humidity 70-76%, Light 603 $\mu E\ m^{-2}\ s^{-1}$ (maximum)). The irradiance of light (day time only) measured in the field and greenhouse showed a constant difference of 4-5 $mmoles.m^{-2}s^{-1}$ throughout the period of analysis. All biochemical analyses were performed in five replicates and repeated twice. Agrometeorological data were obtained from the Indira Gandhi Agriculture University, Raipur, whereas, temperature and relative humidity data for greenhouse were recorded employing a thermohygrometer (Keshavkant and Naithani 2007).

Superoxide was measured as described by Elstner and Heupel (1976) by monitoring the nitrite formation from hydroxylamine in presence of O_2^-. The absorbance in the aqueous solution was read at 530 nm. A standard curve with NO_2^- was used to calculate the production rate of superoxide from the chemical reaction of superoxide and hydroxylamine. The superoxide formation was expressed as $\mu mol.min^{-1}.g^{-1}$fm of the leaf and shoot tissues. Malondialdehyde (MDA) content was measured following the method of Hodges *et al*. (1999) and expressed as $A_{540}.g^{-1}$fm of the leaf and shoot samples.

Leachate conductivity was estimated following the method of Bigras and Calme (1994). Weighed amount of leaf and shoot were cut into 10 mm segments and placed in test tubes filled with 10 ml of distilled water. After 20 hrs at room temperature, the electrical conductivity was measured. The specific conductivity of the leachates was expressed as m $Mhos.g^{-1}$fm of the sample. About 1ml aliquot was collected for analysis of various ions and protein. Protein content in the leachates of leaf and shoot were determined spectrophotometrically (Bradford 1976) and expressed as $mg.g^{-1}$fm of the sample. Potassium (K^+) and calcium (Ca^{++}) ion levels were analyzed in the leachates of leaf and shoot. Both the ions were determined employing the Flame Photometer and were expressed in $mg.g^{-1}$fm of the leaf or shoot.

One way ANOVA and correlation analyses were performed using MS Excel-2003 for showing significant difference between the field and greenhouse sal seedlings, and also to reflect the association of one parameter with the other. The significant differences were calculated at two levels *i.e.* 1 and 5, and expressed as (*) and (**), respectively.

Overproduction of AOS was observed in response to chilling temperature in leaves and shoots of sal seedlings. From June to October, the vigorously growing sal seedlings were characterized by very low rates of superoxide formation in leaves and shoots (Fig. 1). The liberation of superoxide did not enhance further if the seedlings were shifted to greenhouse. Aerial parts of these seedlings showed low rates of superoxide formation during November to March (Fig. 1). In contrast, a significant rise (2 fold, F = 38.48**) was discernible in the superoxide liberation rates in chilling exposed seedlings from November in leaves and shoots and maximum levels were registered in March (Fig. 1).

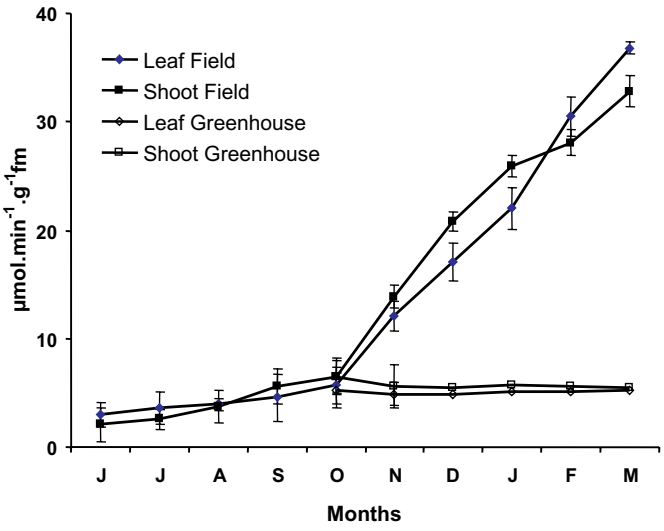

Fig. 1. Relative changes in the liberation of superoxide in the leaves and shoots of sal seedlings during 9 months of growth in field and greenhouse conditions. Each point is the mean ±SE of 5 separate observations.

Changes in the MDA content, a final product of lipid peroxidation, was measured in the leaf and shoot of sal seedlings. Relatively low levels of MDA were recorded in leaf and shoot of one week to 4 month seedlings (Fig. 2). Thereafter, with the fall of air temperature (in field condition), an abrupt increase in the accumulation of MDA was discernible in the aerial parts of nine months seedlings and maximum levels were recorded by the end of March. In contrast, the aerial parts of greenhouse seedlings displayed significantly (F = 7.77*) low rates of MDA accumulation during same period of analysis (Fig. 2).

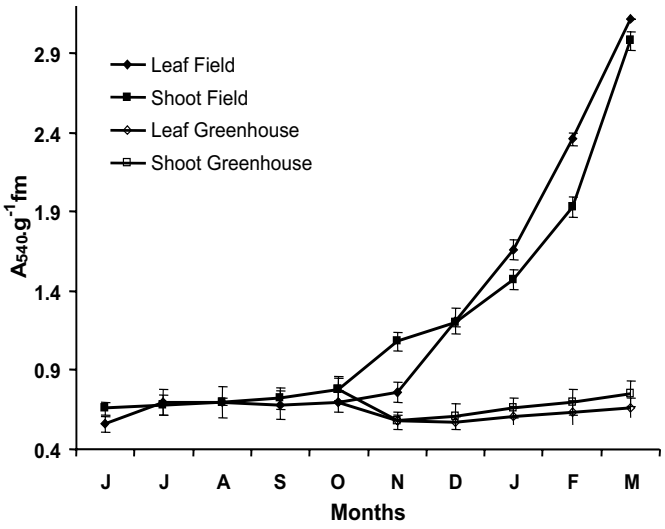

Fig. 2. Accumulation of MDA in leaves and shoots of field and greenhouse maintained sal seedlings over 9 months. Values are mean ±SE of 5 separate replicates.

Initially, from the 2 leaves old to 4 months old seedlings, a gradual rise in conductivity was discernible in the leaves and shoots (Fig. 3a). Later on, with the fall of air temperature from November, a sharp increase (2 fold) in the conductivity was recorded and reached to maximum by March, in leaves and shoots of field grown seedlings (Fig. 3a). On the other side, aerial parts of greenhouse seedlings displayed significantly (F = 13.27**) lower rates of leachate conductivity (Fig. 3a). Similarly, a gradual rise in protein, K^+ and Ca^{++} leakages were recorded in the leaves and shoots (Fig. 3b, 3c and 3d). Later on, with the reduction in air temperature (in field) from November, remarkable increments (1.4-1.8 fold) in leakage of all the above molecules were discernible and reached to maximum by March, both in leaves and shoots (Fig. 3b, 3c and 3d). The leakage of above molecules from the aerial parts of greenhouse seedlings, during chilling period was significantly (F =

www.IndianJournals.com
Members Copy, Not for Commercial Sale
Downloaded From IP - 44.213.60.33 on dated 23-Jul-2024

www.IndianJournals.com
Members Copy, Not for Commercial Sale
Downloaded From IP - 44.213.60.33 on dated 23-Jul-2024

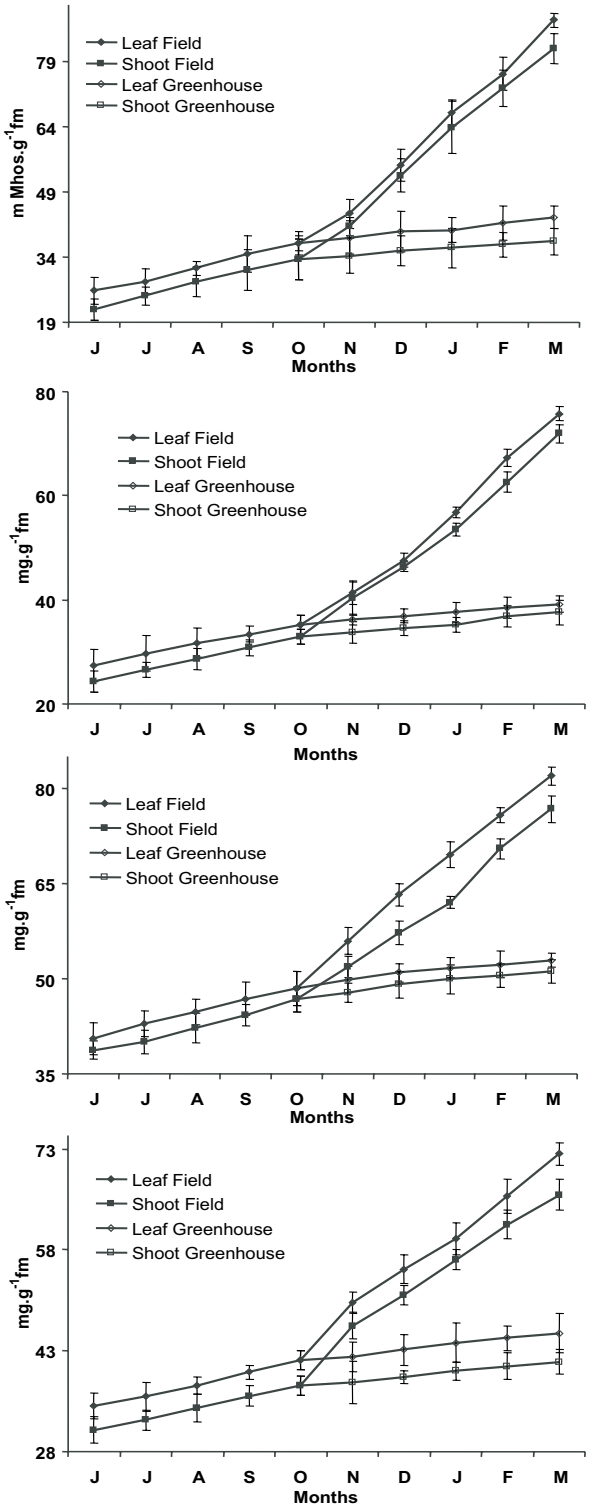

Fig. 3. Temporal changes in the specific conductance (3a) and protein (3b), K+ (3c) and Ca++ (3d) losses from aerial parts of field and greenhouse grown sal seedlings during 9 months. Each value is mean ±SE of 5 replicates.

22.04**) lower than the field grown seedlings (Fig. 3b, 3c and 3d).

The young sal seedlings exposed to chilling (9-14.1^{O}C) constantly for 5 months (November to March) exhibited excessive (5-6 folds) accumulation of superoxide in shoots and leaves (Fig. 1). The overproduction of superoxide has been associated with the chilling injury in these (Keshavkant and Naithani 2007) and other tissues (Lyons 1973). AOS readily oxidize lipids leading to membrane perturbations which in turn could be a potential cause of chilling injury (Lukatkin 2003). Chloroplasts are the primary site for oxidative injury because thylakoid membranes contain a high proportion of polyunsaturated fatty acids, thus highly vulnerable to peroxidation (Leech and Murphy 1976).

In the present study, compared to greenhouse seedlings, the field grown sal seedlings showed remarkable rise (4 fold) in MDA levels both in shoots and leaves (Fig. 2). These increasing rate of MDA production (F = 7.77*) is indicative of altered membrane structure that is responsible for enhanced membrane permeability to ions and proteins. Thus, chilling induced lipid peroxidation has been suggested to play a vital role in conferring irreversible membrane deterioration in chilling sensitive plants leading to severe leakage loss of electrolytes (Hariyadi and Parkin 1993). Lyons (1973) and Paull (1981) have suggested that, loss of membrane integrity results in the enhanced efflux of cellular constituents in the leachates, and is one of the earliest events associated with membrane damage.

The aerial parts of field grown sal seedlings registered a severe leakage loss during exposure to chilling and rate of leakage increased greatly with the extent of chilling exposure (Fig. 3a, 3b, 3c and 3d). Almost 2 fold enhancement in specific conductance were observed in leachates of leaf and shoot (F= 13.27**) of sal seedlings (Fig. 3a) exposed to chilling temperature from November to March, further confirms severity of membrane perturbation due to chilling. Probably, these losses were possible outcomes of the chilling damage and are similar with the observations of Lukatkin (2003) and Apostolova *et al*. (2008). Plants originating in tropical climates have more saturated fatty acids and solidification of these lipids could account for observed death or injury

at low temperatures (Paull 1981). Loss of membrane integrity is tenaciously associated with enhanced leakage loss of various molecules (Chang *et al*. 2001, Apostolova *et al*. 2008). Nearly 2 fold promotion in the protein content was measured in the leachates of leaf and shoot (F = 11.52**) of sal seedlings, during November to March (Fig. 3b). It was well established that the proteins are very much involved in conferring chilling tolerance to the sensitive plants by their capacity to bind free cellular water which are otherwise dangerous to the cells by forming ice crystals (Ismail *et al*. 1999, Uemura *et al*. 2003). Similar results were also reported by Chang *et al*. (2001) and Lukatkin (2003) for *Vigna radiata*, *Zea mays* and *Cucumis sativus* seedlings under chilling stress conditions. Like wise, K^+ and Ca^{++} has been recorded in substantial amounts (F = 22.04**) in leachates of leaves and shoots during chilling injury (Fig. 3c and 3d). The loss of K^+ from damaged aerial parts may inhibit protein synthesis and activity of several K^+ dependant enzymes (Ryyppo *et al*. 1998). Similarly, increased leakage of Ca^{++} may be due to activation of efflux-voltage dependent cation channels (Minorsky 1985). Chilling induced membrane damage followed by leakage of electrolytes from leaf and shoot of field grown sal seedlings were further confirmed by comparing leakage of solutes from the respective tissues of greenhouse seedlings (Fig. 3a, 3b, 3c and 3d). Significantly low levels of specific conductance as well as solutes loss from aerial parts of greenhouse seedlings were indicative of no loss of membrane integrity in these tissues (Fig. 3a, 3b, 3c and 3d).

In conclusion, above data confirms that aerial parts of sal seedlings produced variety of metabolic disorders like excessive generation of AOS, MDA and severe membrane perturbation, evidenced as higher leakage of both organic (protein) and inorganic (K^+ and Ca^{++}) molecules in response to constant exposure to chilling temperatures for 5 months *i.e.* November to March. Thus, it is concluded that over accumulation of AOS and its mediated MDA accumulation leads to membrane perturbation in aerial parts of chilling exposed seedlings ultimately resulting in cell death and mass mortality of sal seedlings.

The authors thank to the Head, School of Life Sciences, Pt. Ravishankar Shukla University, Raipur for providing the necessary facilities. We also acknowledge financial assistance given by the Department of Science & Technology, New Delhi, to carry out this work.

REFERENCES

Apostolova, P., Yordanova, R. and Popova, L. (2008). Response of antioxidant defense system to low temperature stress in two wheat cultivars. *Gen. Appl. Plant Physiol*. **34:** 281-294.

Bigras, F.J. and Calme, S. (1994). Viability tests for estimating root cold tolerance of black spruce seedlings. *Can. J. For. Res.* **24:** 1039-1048.

Bradford, M.M. (1976). A rapid and sensitive method for quantification of microgram quantities of protein utilizing on principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.

Campos, P.S., Quartin, V., Ramalho, J.C. and Nunes, M.A. (2003). Electrolyte leakage and lipid degradation account for cold sensitivity in leaves of Coffea plants. *J. Plant Physiol*. **160:** 283-292.

Chang, M.Y., Chen, S.L., Lee, C.F. and Chen, Y.M. (2001). Cold acclimation and root temperature protection from chilling injury in chilling sensitive mungbean (*Vigna radiata*) seedlings. *Bot. Bull. Acad. Sin*. **42:** 53-60.

Cheour, F., Willemot, C., Arul, J., Makhlouf, J. and Desjardins, Y. (1991). Post harvest response of two strawberry cultivars to foliar application of $CaCl_2$. *Plant Physiol. Hort. Sci.* **26:** 1186-1188.

Elstner, E.F. and Heupel, A. (1976). Inhibition of nitrite formation from hydroxylammoniumchloride: a simple assay for superoxide dismutase. *Anal. Biochem*. **70:** 616-620.

Evans, H.J. and Sorger, G.J. (1966). Role of mineral elements with the emphasis on univalent cations. *Ann. Rev. Plant Physiol*. **17:** 47-76.

Glenn, G.M., Redy, A.S.N. and Pooviah, B.W. (1988). Effect of calcium on wall structure, protein phosphorylation and protein profile in senescing apples. *Plant Cell Physiol*. **29:** 565-572.

Hariyadi, P. and Parkin, K.L. (1993). Chilling induced oxidative stress in cucumber (*Cucumis sativus* L. cv. Calypso) seedlings. *J. Plant Physiol*. **141:** 733-738.

www.IndianJournals.com
Members Copy, Not for Commercial Sale
Downloaded From IP - 44.213.60.33 on dated 23-Jul-2024

Hodges, D.M., DeLong, J.M., Forney, C.F. and Prange, R.K. (1999). Improving the thiobarbituric acid reactive substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta*. **207:** 604-611.

Ismail, A.M., Hall, A.E. and Close, T.J. (1999). Purification and partial characterization of a dehydrin involved in chilling tolerance during seedling emergence of cowpea. *Plant Physiol*. **120:** 237-244.

Karabal, E., Yucel, M. and Oktem, H.A. (2003). Antioxidant responses of tolerant and sensitive barley cultivars to boron toxicity. *Plant Sci.* **164:** 925-933.

Keshavkant, S. and Naithani, S.C. (2007). Low temperature induced changes in the phenolic contents and its regulatory enzymes in sal seedlings. *Indian J. Plant Physiol*. **12:** 146-152.

Kirakosyan, A., Seymour, E., Kaufman, P.B., Warber, S., Bolling, S. and Chang, S.C. (2003). Antioxidant capacity of polyphenolic extracts from leaves of *Crataegus laevigata* and *Crataegus monogyna* subjected to drought and cold stress. *J. Agri. Food Chem*. **51:** 3973-3976.

Lafuente, M.T., Sala, J.M. and Zacarias, L. (2004). Active oxygen detoxifying enzymes and phenylalanine ammonia lyase in the ethylene induced chilling tolerance in citrus fruit. *J. Agri. Food Chem*. **52:** 3606-3611.

Leech, R.M. and Murphy, D.J. (1976). The cooperative function of chloroplasts in the biosynthesis of small molecules. *Top Photosyn*. **1:** 365-401.

Leigh, R.A. and WynJones, R.G. (1984). A hypothesis relating the critical potassium concentration for growth to the distribution and functions of this ion in the plant cell. *New Phytol*. **97:** 1-13.

Lukatkin, A.S. (2003). Contribution of oxidative stress to the development of cold induced damage to leaves of chilling sensitive plants: injury of cell membrane by chilling temperatures. *Russ. J. Plant Physiol*. **50:** 243-246.

Lyons, J.M. (1973). Chilling injury in plants. *Ann. Rev. Plant Physiol*. **24:** 445-466.

Minorsky, P.V. (1985). An heuristic hypothesis of chilling injury in plants: a role for calcium as the primary physiological transducer of injury. *Plant Cell Environ.* **8:** 75-94.

Paull, R.E. (1981). Temperature induced leakage from chilling sensitive and chilling resistant plants. *Plant Physiol*. **68:** 149-153.

Pooviah, B.W. and Leopold, A.C. (1973). Deferral of leaf senescence with calcium. *Plant Physiol*. **52:** 236-239.

Ryyppo, A., Repo, T. and Vappavuori, E. (1998). Development of freezing tolerance in roots and shoots of scots pine seedlings at nonfreezing temperatures. *Can. J. For. Res.* **28:** 257-265.

Saltveit, M.E. (2002). The rate of ion leakage from chilling-sensitive tissue does not immediately increase upon exposure to chilling temperatures. *Post Har. Biol. Technol*. **26:** 295-304.

Sinha, D.N. (1956). Mortality of sal and its associates. Proceedings of IX Silviculture Conference, Manager of Publication, New Delhi, India pp 61-65.

Suzuki, N. and Mittler, R. (2006). Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. *Physiol. Plant*. **126:** 45-51.

Uemura, M., Warren, G. and Steponkus, P.L. (2003). Freezing sensitivity in the sfr4 mutant of *Arabidopsis* is due to low sugar content and is manifested by loss of osmotic responsiveness. *Plant Physiol.* **131:** 1800-1807.

www.IndianJournals.com
Members Copy, Not for Commercial Sale
Downloaded From IP - 44.213.60.33 on dated 23-Jul-2024