Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Protection of ultrastructure in chilling-stressed banana leaves by salicylic acid*

KANG Guo-zhang[1,2], WANG Zheng-xun[†‡3], XIA Kuai-fei[4], SUN Gu-chou[2]

([1]National Engineering Research Centre for Wheat, Henan Agricultural University, Zhengzhou 450002, China)
([2]South China Institute of Botany, Chinese Academy of Sciences, Guangzhou 510650, China)
([3]Department of Biology, Guangzhou University, Guangzhou 510405, China)
([4]College of Life Science, Zhongshan University, Guangzhou 510275, China)
[†]E-mail: wangzhengxun@163.com

Received May 15, 2006; revision accepted Nov. 27, 2006

Abstract: Objective: Chilling tolerance of salicylic acid (SA) in banana seedlings (*Musa acuminata* cv., Williams 8818) was investigated by changes in ultrastructure in this study. Methods: Light and electron microscope observation. Results: Pretreatment with 0.5 mmol/L SA under normal growth conditions (30/22 °C) by foliar spray and root irrigation resulted in many changes in ultrastructure of banana cells, such as cells separation from palisade parenchymas, the appearance of crevices in cell walls, the swelling of grana and stromal thylakoids, and a reduction in the number of starch granules. These results implied that SA treatment at 30/22 °C could be a type of stress. During 3 d of exposure to 7 °C chilling stress under low light, however, cell ultrastructure of SA-pretreated banana seedlings showed less deterioration than those of control seedlings (distilled water-pretreated). Conclusion: SA could provide some protection for cell structure of chilling-stressed banana seedling.

Key words: Banana (*Musa acuminata* cv., Williams 8818), Chilling tolerance, Salicylic acid, Ultrastructure
doi:10.1631/jzus.2007.B0277 **Document code:** A **CLC number:** S668

INTRODUCTION

Banana plants, which are very sensitive to chilling stress, are often damaged seriously by chilling episodes frequently occurring during winter or early spring every year in South China. Thus, it is important to look for different ways to improve the chilling tolerance of banana plants. To our knowledge, however, only a few such studies on these episodes have been reported (Liang *et al*., 1994).

Salicylic acid (SA) is a natural signaling molecule, mediating resistance in response to avirulent pathogens. In plants, SA is endogenously synthesized, and plays an essential role in the defense against pathogen attack (Cronje and Bornman, 1999; Ding *et al*., 2001; Mayer *et al*., 2005; Rajjou *et al*., 2006). Although past focus has been mainly on the role of SA on biotic stresses, several recent studies showed that SA can enhance plant resistance to environmental stresses, such as ultraviolet light, drought, salt, chilling and heat (Yalpani *et al*., 1994; Dat *et al*., 1998; Mishra and Choudhuri, 1999; Janda *et al*., 1999; Senaratna *et al*., 2000; He *et al*., 2005; Ogawa *et al*., 2005). Our previous studies found that SA pretreatment enhanced the chilling tolerance of banana seedlings. In its physiological, chemical, and molecular bases, we found that SA inhibited leakage of electrolytes, increased the activities of cell protective enzymes, reduced the accumulation of actived oxygen species (AOS), enhanced efficiency of photosynthesis, and induced high expression of selected genes during chilling treatment (Kang *et al*., 2003a; 2003b; 2004).

Since chilling injury can also be manifested in

‡ Corresponding author
* Project (No. 30471112) supported by the National Natural Science Foundation of China

the ultrasturctural level (Lyons, 1973; Murata, 1990; Kratsch and Wise, 2000), the present study was undertaken to evaluate the alteration of cell ultrastructure by SA in relation to its chilling tolerance in banana seedling plants. And the results provided evidence of enhancing chilling tolerance induced by SA in banana seedlings via the cellular ultrastructure.

MATERIALS AND METHODS

Plant materials

Tissue culture seedlings of banana (*Musa acuminata* cv., Williams 8818, a chilling-sensitive cultivar) grown in plastic pots with soil and sand mixture (10:1, v/v) were used for this study. Seedlings were placed in plastic growth chamber under normal growth conditions [30/22 °C day/night, 75% relative humidity (RH), 12-h photoperiod with a PPFD of 250 μmol/(m^2·s)] and supplied with the same levels of water and MS nutrient. Uniform and healthy seedlings of about two month old (11 cm high) were selected for the experiments.

SA treatment and chilling stress

The above banana seedlings were pretreated by spraying leaf-blades with SA (0.5 mmol/L in distilled water) 1 d before chilling stress with an atomizer until it ran off. At the same time, 10 ml of the SA solution was used to irrigate roots of each plant. Distilled water was applied to leaves and roots of control plants. Then, SA-pretreated and control were transferred to a climatic chamber [a PPFD of 150 μmol/(m^2·s), a 12-h photoperiod and 70% RH] and subjected to chilling stress at 7 °C, which was a temperature of chilling stress but no cold necrosis appeared in leaves (Kang *et al*., 2003a). After 3 d of exposure to 7 °C chilling stress, the seedlings were returned to the previous growth conditions to recover for an additional 2 d. Preliminary experiments showed that treatment with 0.2 mmol/L SA or lower was not reproducibly effective, while higher concentrations (higher than 0.9 mmol/L) caused visible damage (more area of wilting or chilling necrosis) to banana seedlings (Kang *et al*., 2003b). For this reason, this study was focused on SA 0.5 mmol/L.

Light and electron microscope observation

After chilling treatment, four seedlings from each treatment were randomly selected for cell ultrastructural studies. Leaves were collected after 1 d of SA 0.5 mmol/L water solution treatment at 30/22 °C, and after 3 d of chilling stress at 7 °C. Samples from the middle portion of the last developed leaf blades were excised and fixed in cold 2% (v/v) glutaraldehyde in 0.1 mol/L potassium phosphate buffer (PBS, pH 7.2), vacuum-infiltrated until the material sank and was left overnight at 4 °C. Samples were then washed in five changes of 0.1 mol/L PBS buffer (20 min each) and postfixed in 1% OsO_4 for 16 h at 4 °C in 0.1 mol/L PBS buffer. The materials were washed with five changes of 0.1 mol/L PBS buffer, dehydrated in a graded ethanol series, and embedded in Epon812 resin. Ultra-thin sections about 80~90 nm were obtained with a diamond knife on a Leica-Ultracut S ultramicrotome, and then stained with 2% uranyl acetate for 90 min and 6% lead citrate for 10 min. Sample semimicrosections of 0.2 μm were made with Pyramitome of LKB11800, and finally examined in an OLYMPUS A 70 light microscope and a JEM-1010 (Japan) transmission electron microscope at 90 kV, respectively. At least five sections from each treatment were examined.

RESULTS AND DISCUSSION

At light microscopic level, it was observed that the cells of palisade parenchymas in leaves of control plants arranged in a regular pattern (Figs.1a and 1e). Treatment with 0.5 mmol/L SA at 30/22 °C for 1 d resulted in the separation of some cells from their regular sites (Figs.1b and 1f). After 3 d of 7 °C stress, most of the cells in control seedlings separated from each other, indicating that the disassembly of palisade parenchymas occurred in this case (Figs.1c and 1g). However, not much alteration of cell arrangement in palisade parenchymas was found in chilling stress banana seedlings with SA-pretreatment (Figs.1d and 1h). It could perhaps be speculated that alleviated changes by SA in cell structure occurred under chilling stress.

At light electron level, it was further found that chloroplasts in mesophyll cells of control plants at 30/22 °C had many starch granules (2.5 granules in each chloroplast) (Fig.2a) and that well-developed granal stacks were interconnected by stromal

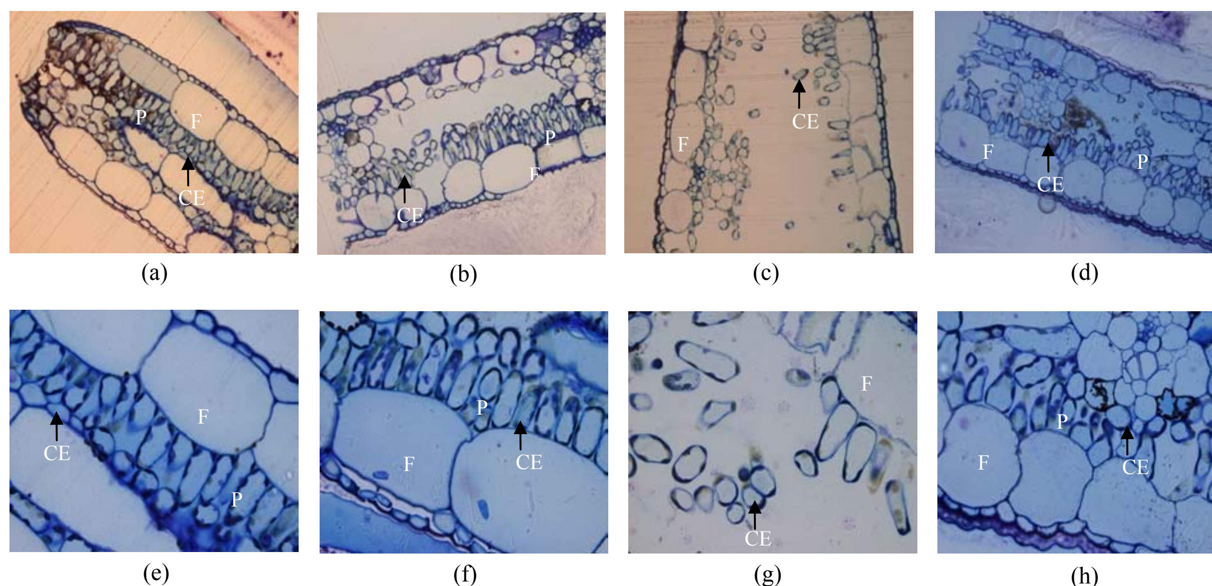

Fig.1 Light microscopy observation for effects of SA on cell structure in chilling-stressed banana seedling leaves. (a) Mesophyll cells of banana seedlings growing at 30/22 °C (10×40); (b) Mesophyll cells of banana seedlings treated by 0.5 mmol/L SA at 30/22 °C for 1 d (10×40); (c) Mesophyll cells of banana seedlings suffered from 7 °C stress for 3 d (10×40); (d) Mesophyll cells of SA-pretreated banana seedlings suffered from 7 °C stress for 3 d (10×40); (e) Mesophyll cells of banana seedlings growing at 30/22 °C (10×100); (f) Mesophyll cells of banana seedlings treated by 0.5 mmol/L SA at 30/22 °C for 1 d (10×100); (g) Mesophyll cells of banana seedlings suffered from 7 °C stress for 3 d (10×100); (h) Mesophyll cells of SA-pretreated banana seedlings suffered from 7 °C stress for 3 d (10×100)
CE: Cell; F: Fibro; P: Palisade parenchymas

thylakoids (Figs.2e and 2i). The chloroplast membranes were intact (Figs.2e and 2i), as were most of mitochondrial membranes (Figs.2m). Whereas, after the treatment with SA at 30/22 °C for 1 d, swelling and separation of 40% of chloroplant thylakoids occurred, which resulted in an increase in intrathylakoid space (Figs.2f and 2j). At the same time, most of the starch granules disappeared in chloroplasts (0.2 granules in each chloroplast), and unexpectedly, there appeared 11~12 crevices in each cell wall (Fig.2b), and part of about 20% mitochondrial membranes were broken and the mitochondrial matrix might mix with cytoplasm (Fig.2n).

It had been reported that chloroplasts are commonly the earliest visible site of ultrastructural chilling injury in plant cells (Kratsch and Wise, 2000). Three days of 7 °C stress caused a series of pronounced changes in the fine structure of cells both in control and SA-pretreated banana plants. In control chloroplasts, almost all of the starch granules were lost (Fig.2c), and 90% granal and stromal thylakoids became enlarged, and presented some large vesicles in chloroplast granal and stromal thylakoids (Figs.2g and 2k). The mitochondrial cristae remained intact, but some membranes were broken-down (Fig.2o). In the cells of SA-pretreated leaves subjected to 7 °C, however, more starch granules were found in chloroplasts (1~2 granules in each chloroplast), and the number of crevices in each cell wall decreased to 3~4 trips (Fig.2d). Although most of the granal and stromal thylakoids became swollen, they still remained in the ordered state (Figs.2h and 2l). Moreover, mitochondrial membranes were kept more intact than those in the control cells in this case (Figs.2o and 2p).

In the present study, the unordered arrangement of cells, swelling of granal and stromal thylakoids (Figs.1c and 1g, and Figs.2g and 2k) in control plants under chilling condition, suggested that leaves of banana plants were very sensitive to chilling stress. However, the less ultrastructural deterioration in SA-pretreated plants (Figs.1d, 1h, 2h and 2l) indicated that SA might provide protection for banana seedlings to chilling injury and could increase the chilling tolerance of banana seedlings. On the contrary, a series of degradative changes in cell structures after SA

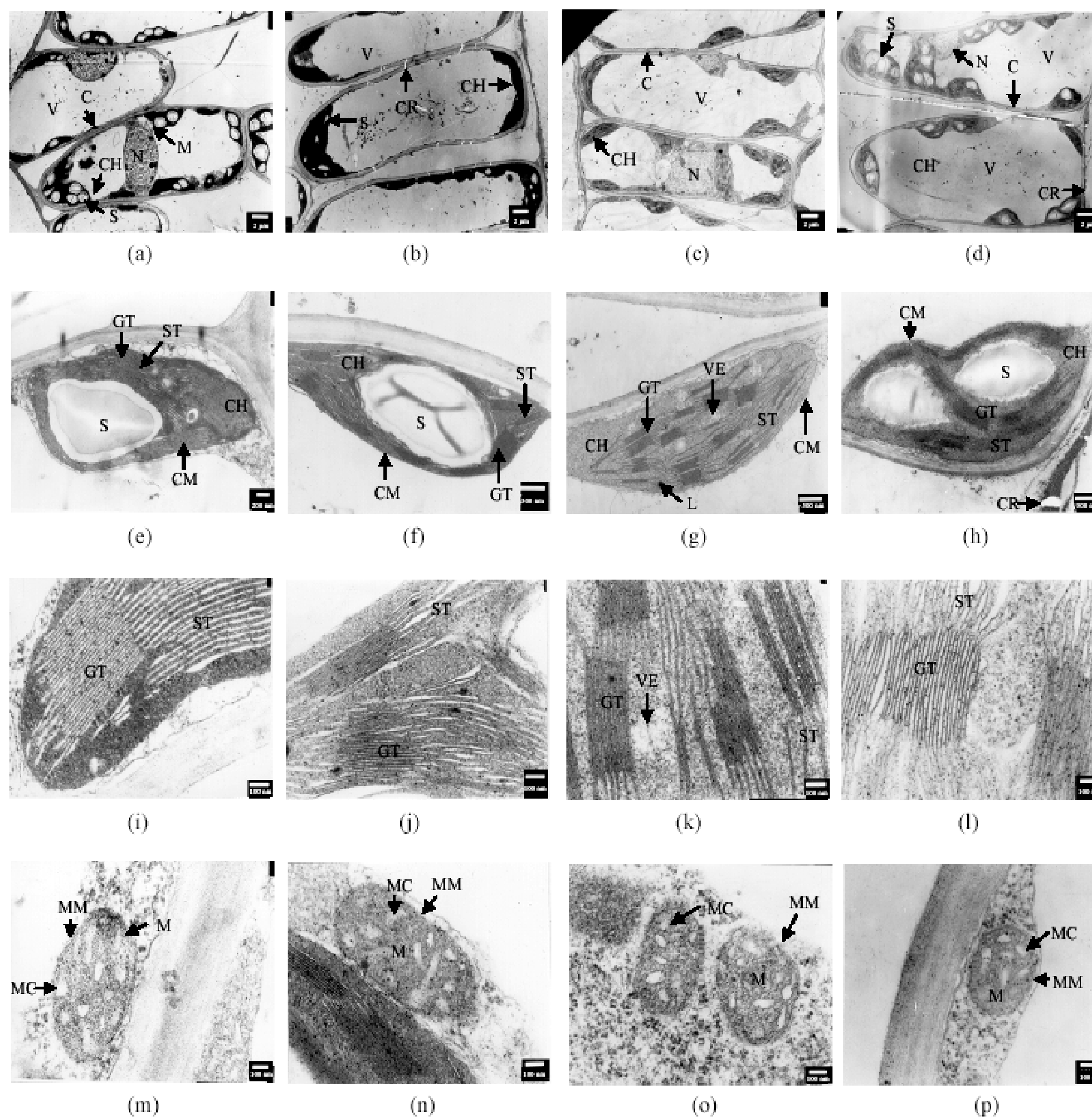

Fig.2 Electron microscope observation for effects of SA on ultrastructure in chilling-stressed banana seedling leaves. (a) Mesophyll cells of banana seedlings growing at normal growth conditions (30/22 °C); (b) Mesophyll cells of banana seedlings treated by 0.5 mmol/L SA at 30/22 °C for 1 d; (c) Mesophyll cells of banana seedlings suffered from 7 °C stress for 3 d; (d) Mesophyll cells of SA-pretreated banana seedlings suffered from 7 °C stress for 3 d; (e) Chloroplast of mesophyll cells of banana seedlings growing at 30/22 °C; (f) Chloroplast of mesophyll cells of banana seedlings treated by SA at 30/22 °C for 1 d; (g) Chloroplast of mesophyll cells of banana seedlings suffered from 7 °C stress for 3 d; (h) Chloroplast of mesophyll cells of SA-pretreated banana seedlings suffered from 7 °C stress for 3 d; (i) Thylakoid of mesophyll cells of banana seedlings growing at 30/22 °C; (j) Thylakoid of mesophyll cells of banana seedlings treated by SA at 30/22 °C for 1 d; (k) Thylakoid of mesophyll cells of banana seedlings suffered from 7 °C stress for 3 d; (l) Thylakoid of mesophyll cells of SA-pretreated banana seedlings suffered from 7 °C stress for 3 d; (m) Mitochondria of mesophyll cells of banana seedlings growing at 30/22 °C; (n) Mitochondria of mesophyll cells of banana seedlings treated by SA at 30/22 °C for 1 d; (o) Mitochondria of mesophyll cells of banana seedlings suffered from 7 °C stress for 3 d; (p) Mitochondria of mesophyll cells of SA-pretreated banana seedlings suffered from 7 °C stress for 3 d

C: Cell membrane; CH: Chloroplast; CM: Chloroplast membrane; CR: Crevice; GT: Grana thylakoid; L: Lipid droplet; M: Mitochondria; MC: Mitochondria cristae; MM: Mitochondria membrane; N: Nucleus; S: Starch; ST: Stromal thylakoid; V: Vacuole; VE: Vesicle. White lines of black panes express bars of magnification

treatment at 30/22 °C were quite striking (Figs.1b, 1f, 2f, 2j, 2n), suggesting that SA treatment under normal growth conditions could be a type of stress. Some researchers have hypothesized that there is a common denominator, e.g. when stress treatments are applied at mild levels that do not cause appreciable damage (Janda *et al*., 1999). According to this, it was proposed that plants respond with similar defense systems to a wide range of stresses such as chemical pollutants, UV radiation, low oxygen, pathogen infection, and wounding (Ding *et al*., 2001). Moreover, the enhanced chilling tolerance in drought-hardened maize plants also supported this view (Scandalios, 1993). Thus, we deduced that treatment of banana seedlings with 0.5 mmol/L SA at 30/22 °C could be a mild stress and indirectly or directly induce some of the cell defense mechanism responses providing protection against subsequent chilling stress, rather than producing a direct effect by SA itself.

SA pretreatment under normal growth conditions damaged thylakoid membranes of banana chloroplasts, etc. (Figs.2f and 2j), which were supported by the decrease in starch granules (Fig.2b), and were in accordance with low photosynthetic activity (Kang *et al*., 2003b). Moreover, more starch granules and intactness of cell ultrastructure in SA-pretreated leaves during chilling stress (Figs.2d, 2h and 2l) might be linked to their beneficial photosynthetic function (Kang *et al*., 2003b).

Clearly, the appearance of numerous crevices in cell wall after SA treatment at 30/22 °C and reduction during chilling treatment were interesting. It is hard to make a relevant explanation right now. We first guessed that crevices, which appeared in SA pretreatment at normal growth conditions, might be from the toxicity of AOS (such as H_2O_2) induced by SA. The reason for this supposition was that exogenous SA treatments led to an increase in H_2O_2 levels in plant tissues (Lamb and Dixon, 1997; Kang *et al*., 2003a), and, in consequence, accumulated AOS could destroy cell structures (Prasad, 1996). However, no crevice appeared in control plants after 3 d of 7 °C chilling stress, in which probably more AOS accumulated (Kang *et al*., 2003a), and crevices in the cell walls of SA-pretreated banana became fewer at this time. So, it seemed that oxidative destruction would not be the exact reason for cell wall crevice formation. Perhaps, some effect of SA on cell walls would take place during their synthesis or expansion. Primary cell wall expansion requires a loosening of the crosslinks within the wall and an increase in cytoplasmic pressure potential. Under normal growth conditions, the destroyed cell structure might increase cytoplasmic pressure potential, and tear the cell walls, to form the crevices. Under chilling stress conditions, SA was also reported to enhance the activity and expression of alterative oxidase in plants (Gilliland *et al*., 2003), which could increase heat production (Rasin *et al*., 1987; van der Straeten *et al*., 1995). Therefore, during chilling stress, heat production mediated through alterative respiration pathway by SA might play a role in protection against chilling and repairing the crevices of cell walls.

In conclusion, SA treatment under normal growth conditions could be a mild stress, but enhance the tolerant capability of banana cells to subsequent chilling stress.

We would like to express our appreciation to Prof. Zhifang Lin for help with the electron microscopy and modification of manuscript.

References

Cronje, M.J., Bornman, L., 1999. Salicylic acid influences Hsp70/Hsc70 expression in *Lycopersicon esculentum*: dose- and time-dependent induction or potentiation. *Biochem. Biophys. Res. Commun.*, **265**(2):422-427. [doi:10.1006/bbrc.1999.1692]

Dat, J.F., Lopez-Delgado, H., Foyer, C.H., Scott, I.M., 1998. Parallel changes in H_2O_2 and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings. *Plant Physiol.*, **116**(4):1351-1357. [doi:10.1104/pp.116.4.1351]

Ding, C.K., Wang, C.Y., Gross, K.C., Smith, D.L., 2001. Reduction of chilling injury and transcript accumulation of heat shock proteins in tomato fruit by methyl jasmonate and methyl salicylate. *Plant Sci.*, **161**(6): 1153-1159. [doi:10.1016/S0168-9452(01)00521-0]

Gilliland, A., Singh, D.P., Hayward, J.M., Moore, C.A., Murphy, A.M., York, C.J., Slator, J., Carr, J.P., 2003. Genetic modification of alternative respiration has differentia effects on antimycin A-induced versus salicylic acid-induced resistance to *Tobacco mosaic virus*. *Plant Physiol.*, **132**(3):1518-1528. [doi:10.1104/pp.102.017640]

He, Y.L., Liu, Y.L., Cao, W.X., Huai, M.F., Xu, B.G., Huang, B.R., 2005. Effects of salicylic acid on heat tolerance associated with antioxidant metabolism in *Kentucky Bluegrass*. *Crop Sci.*, **45**(3):988-995. [doi:10.2135/cropsci2003.0678]

Janda, T., Szalai, G., Tari, I., Paldi, E., 1999. Hydroponic treatment with salicylic acid decreases the effects of

chilling injury in maize (*Zea mays* L.) plants. *Planta*, **208**(2):175-180. [doi:10.1007/s004250050547]

Kang, G.Z., Wang, C.H., Sun, G.C., Wang, Z.X., 2003a. Salicylic acid changes activities of H_2O_2-metabolizing enzymes and increases the chilling-tolerance of banana seedlings. *Environ. Exp. Bot.*, **50**:9-15.

Kang, G.Z., Ou, Z.Y., Sun, G.C., Wang, Z.X., 2003b. Effects of salicylic acid on cell membranes and some photosynthesis of chilling-stressed banana seedlings. *Acta Hort. Sin.*, **30**(2):141-146 (in Chinese).

Kang, G.Z., Zhu, G.H., Peng, X.X., Sun, G.C., Wang, Z.X., 2004. Isolations of salicylic acid-induced genes in chilling-stressed banana seedling leaves using mRNA differential display. *J. Plant Physiol. Mol. Biol.*, **30**(2):225-228.

Kratsch, H.A., Wise, R.R., 2000. The ultrastructure of chilling stress. *Plant Cell Environ.*, **23**(4):337-350. [doi:10.1046/j.1365-3040.2000.00560.x]

Lamb, C., Dixon, R.A., 1997. The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **48**(1):251-275. [doi:10.1146/annurev.arplant.48.1.251]

Liang, L.F., Wang, Z.H., Zhou, B.Y., Huang, H.B., 1994. Effects of low temperature and paclobutrazol on the activities and isozymes of peroxidase in banana leaves. *J. South China Agric. Univ.*, **15**(3):65-70 (in Chinese).

Lyons, J.M., 1973. Chilling injury in plants. *Annu. Rev. Plant Physiol.*, **24**(1):445-466. [doi:10.1146/annurev.pp.24.060173.002305]

Mayer, C.M., Lee, K.C., Moore, C.A., Wong, S.M., Carr, J.P., 2005. Salicylic acid-induced resistance to *Cucumber mosaic vivus* in *Squash* and *Arabidopsis*: contrasting mechanisms of induction and antiviral action. *Mol. Plant Microbe Interact.*, **18**(5):428-434.

Mishra, A., Choudhuri, M.A., 1999. Effects of salicylic acid on heavy metal-induced membrane deterioration mediated by lipoxygenase in rice. *Biol. Plant.*, **42**(3):409-415. [doi:10.1023/A:1002469303670]

Murata, T., 1990. Relation of Chilling Stress to Membrane Permeability. *In*: Wang, C.Y. (Ed.), Chilling Injury of Horticultural Crops. CRC Press, Boca Raton, Florida, p.201-209.

Ogawa, D., Nakajima, N., Sano, T., Tamaoki, M., Aono, M., Kwbo, A., Kanna, M., Ioki, M., Kamada, H., Saji, H., 2005. Salicylic acid accumulation under O_3 exposure is regulated by ethylene in tobacco plants. *Plant Cell Physiol.*, **46**(7):1062-1072. [doi:10.1093/pcp/pci118]

Prasad, T.K., 1996. Mechanisms of chilling-induced oxidative stress injury and tolerance: changes in antioxidant system, oxidation of proteins and lipids and protease activities. *Plant J.*, **10**(6):1017-1026. [doi:10.1046/j.1365-313X.1996.10061017.x]

Rajjou, L., Belghazi, M., Huguet, R., Robin, C., Moreau, A., Job, C., Job, D., 2006. Preteomic investigation of the effect of salicylic acid on Arabidopsis seed germination and establishment of early defense mechanisms. *Plant Physiol.*, **141**(3):910-923. [doi:10.1104/pp.106.082057]

Rasin, I., Ehmann, A., Melander, W.R., Meeuse, B.J.D., 1987. Salicylic acid: a natural inducer of heat production in *Arum. lilies*. *Science*, **237**:1601-1602.

Scandalios, J.G., 1993. Oxygen stress and superoxide dismutases. *Plant Physiol.*, **101**(1):7-12.

Senaratna, T., Touchell, D., Bunn, T., Dixon, K., 2000. Acetyl salicylic acid (aspirin) and salicylic acid induce multiple stress tolerance in bean and tomato plants. *Plant Growth Reg.*, **30**(2):157-161. [doi:10.1023/A:1006386800974]

van der Straeten, D., Chaerle, L., Sharkov, G., Lamlers, A., van Montagu, M., 1995. Salicylic acid enhances the activity of the alternative pathway of respiration in tobacco leaves and induces thermogenicity. *Planta*, **196**(3):412-419. [doi:10.1007/BF00203637]

Yalpani, N., Enyedi, A.J., Leon, J., Raskin, I., 1994. Ultraviolet light and ozone stimulate accumulation of salicylic acid, pathogen-related proteins and virus resistance in tobacco. *Planta*, **193**(3):372-376. [doi:10.1007/BF00201815]

Editors-in-Chief: Wei YANG & Peter H. BYERS
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online), monthly

Journal of Zhejiang University
SCIENCE B

www.zju.edu.cn/jzus; www.springerlink.com
jzus@zju.edu.cn

JZUS-B **focuses on "Biomedicine, Biochemistry & Biotechnology"**

JZUS-B **online in PMC:** http://www.pubmedcentral.nih.gov/tocrender.fcgi?journal=371&action=archive