A Trifloxystrobin Fungicide Induces Systemic Tolerance to Abiotic Stresses

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Trifloxystrobin is a strobilurin fungicide, which possesses broad spectrum control against fungal plant diseases. We demonstrated that pre-treating red pepper plants with trifloxystrobin resulted in increased plant growth and leaf chlorophyll content compared with those in control plants. Relative water content of the leaves and the survival rate of intact plants indicated that plants acquired systemic tolerance to drought stress following trifloxystrobin pre-treatment. The recovery rate by re-hydration in the drought treated plant was better in those pre-treated with trifloxystrobin than that in water treated plants. Induced drought tolerance activity by trifloxystrobin was sustained for 25 days after initial application. The trifloxystrobin treated red pepper plants also had induced systemic tolerance to other abiotic stresses, such as frost, cold, and high temperature stresses. These findings suggest that applying the chemical fungicide trifloxystrobin induced systemic tolerance to certain abiotic stresses in red pepper plants.

Keywords : cold and freezing stresses, drought stress, high temperature stress, induced systemic tolerance, plant growth promotion

Plants rely on many adaptive strategies against different abiotic and biotic stresses because of their immobility. Applications of certain chemicals and plant associated microbes are known to induce resistance pathways to abiotic and biotic stresses (Sticher et al., 1997; Schreiber and Desveaux, 2008; Yang et al., 2010). In systemic acquired resistance (SAR), plants develop a broad spectrum of systemic resistance against plant diseases or abiotic stresses upon pathogen attack or treatment of chemicals (Ryals et al., 1996). Root colonization by biocontrol microbes also elicits systemic resistance, known as induced systemic resistance (ISR), against abiotic stresses and a

broad spectrum of diseases caused by bacterial, fungal, and viral pathogens (Kim et al., 2011).

Recent studies suggest that certain chemical applications can induce systemic resistance against abiotic or biotic stresses (Schreiber and Desveaux, 2008). Plants treated with one chemical inducer, β -aminobutyric acid (BABA), show dual induction for resistance to abiotic and biotic stresses (Ton et al., 2005). Application of strobilurin to tobacco induces resistance against *Tobacco Mosaic Virus* and wildfire diseases (Herms et al., 2002). Soil application of a chemical pesticide, imidacloprid, reduces the incidence of citrus canker disease (Graham and Myers, 2011). Induction of drought stress tolerance in plants occurs with the fungicides, ketoconazole and propiconazole, and with the insecticide, imidacloprid (Abdul Jaleel et al., 2007b; Han et al., 2010; Manivannan et al., 2007). Soil applications of propiconazole also induce tolerance to drought and induce tolerance to salinity (Adul Jallel et al., 2008), and imidacloprid-treated plants are tolerant to high temperature (Gonias et al., 2008). Induced tolerance to flooding and salinity occurs with treatments with the plant regulator paclobutrazol (Abdul Jaleel et al., 2007c; Lin et al., 2005).

Trifloxystrobin, a strobilurin fungicide, is used to control a wide range of plant diseases because of its broad spectrum antifungal activity (Reuveni et al., 2000). Trifloxystrobin blocks electron transfer and inhibits mitochondrial respiration in the target fungus (Bartlett et al., 2002). In this study, we examined whether trifloxystrobin could induce systemic tolerance against the abiotic stresses of drought, low temperature, freezing, and high temperature in red pepper plants. We also examined the growth promotion effects of trifloxystrobin and induced systemic resistance to plant diseases.

Effect of trifloxystrobin pretreatment on plant growth and chlorophyll content. Surface-sterilized red pepper seeds were planted in pots to determine the effect of a suspension concentrate type formulation of trifloxystrobin (SC) on plant growth and photosynthesis. Trifloxystrobin SC was obtained from Bayer Cropscience Korea and was

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diluted with water to 1,000 × (double dose), 2,000 × (standard dose), and 4,000 × (half dose). A standard dose of azoxystrobin SC was used as the reference, and the same volume of water was used as a negative control. We used azoxystrobin as a control fungicide, because azoxystrobin is the world's largest selling strobilurin fungicide (Bartlett et al., 2002).

Red peppers (cv. Bukwang) were grown in a soil-less mixture. All seeds were surface-sterilized by soaking them in 70% ethanol for 2 min and 1% sodium hypochlorite for 20 min. After an extensive wash with sterile distilled water, the seeds were planted into sterile soil-less medium (BioHorticulture soil:vermiculite, 7:3, v/v) in a small rectangular pot with 1 seed/pot. These pots were watered with 20 ml of sterile water every 2 days. Seedlings were grown with a cycle of 16 h of light and 8 h of dark under 40-W fluorescent lights (2,000 lux, 80 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). Temperature was maintained at $25 \pm 1^\circ\text{C}$ with a relative humidity of 50–60%. Ten days after planting, the seedlings were transferred to a plastic pot (90 × 90 × 90 mm), and the same growth conditions were maintained for additional three weeks before the chemical treatments.

Pot experiments were conducted in a greenhouse located at Chonnam National University. The chemical solutions (5 ml/plant) were applied in a foliar spray to evaluate induced tolerance against environmental stresses, and by root drenching to evaluate induced plant disease resistance. As controls, distilled water and the reference chemical fungicides were applied to leaves by spray or applied to the

roots. One week after the chemical fungicide treatment, the red pepper plants were exposed to various environmental stresses and plant pathogens.

Chemicals or sterile water as a control treatment were applied by root drenching. Six weeks after application of the chemical fungicides, chlorophyll content was measured in at least 15 different leaves using a portable chlorophyll meter SPAD-502 (Minolta Co., Tokyo, Japan) and the heights of the red pepper plants were measured. Chlorophyll content in leaves of the 2,000 × and 4,000 × diluted trifloxystrobin SC-treated red pepper plants was significantly higher than that in control or the reference chemical fungicide-treated red peppers (Fig. 1A). No effects were observed at the 1,000 × dilution. The height of the red pepper plants treated with 4,000 × diluted trifloxystrobin SC increased significantly compared to those of other treatments and the control (Fig. 1B). The growth of red pepper was promoted at the standard dose (2,000 × diluted) of reference chemical fungicides. Certain strobilurin fungicides have been reported to induce physiological and metabolic alterations; in wheat, kresoxym-methyl inhibits ethylene biosynthesis and increases cytokinin, possibly explaining the delay in senescence and extension of green pigmentation in leaves of fungicide-treated plants (Grossman and Retzlaff, 1997). Application of the triazole fungicides triadimefon and hexaconazole to white yam inhibits amylase and increases starch and sugar contents, and, consequently, the weight (Abdul Jaleel et al., 2007a). The mechanisms underlying the increased growth and chlorophyll effects caused by trifloxystrobin SC await elucidation.

Fig. 1. Effect of trifloxystrobin root drenching on chlorophyll content (A) and height of red pepper (B). Defined dilutions of trifloxystrobin suspension concentrates (SC), azoxystrobin SC, and water as a negative control were applied to red pepper seedlings by root drenching. Six weeks after root drenching, chlorophyll content and height of the red peppers were measured. Chlorophyll content of the red pepper leaves was measured using a portable chlorophyll meter. Each bar point represents the mean $\pm$ standard deviation from three replicate experiments each with 15 plants/treatment. Bars with different letter superscripts are statistically different at $P < 0.05$.

Fig. 2. Effect of trifloxystrobin suspension concentrate (SC) treatment on induction of drought tolerance in red pepper. Pepper plants were grown in pots with soil-less medium and were treated with defined concentrations of trifloxystrobin SC, azoxystrobin SC, or water as a negative control. Five days after treatment, a drought stress was employed by withholding water for ten days, and then relative water content of the red pepper seedlings was measured (A). Two days after rehydration of the drought-stressed red pepper, surviving seedlings were counted to evaluate induced drought tolerance (B). Each bar represents the mean $\pm$ standard deviation of three replicate experiments with 15 plants/treatment. Bars with different letter superscripts are statistically different at $P < 0.05$.

Induced systemic tolerance by trifloxystrobin to various abiotic stresses. To examine induce drought tolerance in the chemical treated plants, the defined chemical dosages or water were pre-treated for five days with regular watering at two days intervals, and then water was withheld for ten days. The plants were rehydrated with sterile water and recovery rate was estimated after two days. To measure water status, plant leaves were cut from pot-grown plants for ten days after drought stress and the relative water content (RWC) was determined to assess severity of the drought stress. Excised leaves cut from the whole stem were weighed (FW) and exposed to distilled water in the dark at 4°C for 24 h and weighed to determine fresh turgid weight (FTW). The leaves were transferred to a paper envelope and dried at 65°C for 24 h, and the desiccated sample was weighed to determine dry weight (DW). RWC was calculated from these measurements as follows: $RWC = (FW - DW) / (FTW - DW)$.

Foliar applications of trifloxystrobin SC to red pepper plants induced tolerance against drought stress (Fig. 2). Treatments with standard and double doses of trifloxystrobin SC resulted in the highest survival rates following drought stress. Significantly higher RWC values were observed in red peppers treated with the standard dose and double dose of trifloxystrobin SC, compared with red pepper plants treated with water (Fig. 2A). Significant drought stress protection was also observed in red pepper plants that were treated with the half dose of trifloxystrobin SC and the standard dose of another reference chemical fungicide, but

to a lesser extent (Fig. 2B). The tolerance induced by the trifloxystrobin SC treatment was maximum at five days after treatment and lasted until twenty-five days after treatment (data not shown).

Five days after treatments, the red pepper plants were placed at -8°C for 30 minutes to examine responses to freezing and for cold stress the exposure was -1°C for 9 or 24 h. The plants were returned to room temperature for two days to measure recovery based on survival. Pre-treatment with the standard dose (2,000 $\times$ dilution) or double dose (1,000 $\times$ dilution) of trifloxystrobin SC induced tolerance to cold stress (Fig. 3A and 3B) and to freezing stress (Fig. 3C and 3D), the effect was dose dependent (Fig. 3A–3D).

Foliar applications of trifloxystrobin SC to red pepper plants resulted in induced tolerance against high temperature (Fig. 4). Treatments with the standard dose of trifloxystrobin SC showed the highest RWC with the high temperature stress for 1 day at 42°C . Significant high temperature stress protection was also observed in red pepper plants that were treated with half and double doses of trifloxystrobin SC (Fig. 4). Additionally, a significantly higher RWC was observed in red peppers treated with the standard dose of the reference chemical fungicide, but to a lesser extent than that of the trifloxystrobin SC-treated red peppers (Fig. 4).

Induced systemic resistance by trifloxystrobin was studied in red pepper challenged with *Xanthomonas campestris* pv. *vesicatoria*, and tobacco (cv. Xanthi) plants exposed to Tobacco Mosaic Virus. Red peppers were grown for 3 weeks and the chemical fungicides or water as a negative

Fig. 3. Induction of cold (A and B) and frost (C and D) stress tolerance in red pepper following foliar application of trifloxystrobin suspension concentrates (SC). Three-week-old red peppers grown in pots were treated with foliar applications of defined concentrations of trifloxystrobin SC, azoxystrobin SC, and water as a negative control. Five days after the application, the plants were exposed to -1°C for 9 h for cold stress and 30 minutes at -8°C for freezing stress before returning to room temperature. A and C, Survival rates after cold or frost stresses were measured by counting the number of plants that survived the treatments. B and D, Representative photographic images of red pepper plants following induced cold or frost tolerance by trifloxystrobin SC are shown. Each bar represents the mean $\pm$ standard deviation of three replicate experiments with 15 plants/treatment. Bars with different letter superscripts are statistically different at $P < 0.05$.

control were applied by root drenching as described above. For pathogen challenge, a bacterial pathogen, *X. campestris* pv. *vesicatoria* 11158, provided by the Korean Agriculture Culture Collection, was cultured at 28°C for 48 hr in tryptic soy agar medium (Difco Inc., Detroit, MI, USA), and then the culture was re-suspended in 0.85% NaCl and adjusted to 1×10^8 colony forming units/ml ($\text{OD}_{600\text{nm}} = 0.2$). The bacterial suspension was mixed with silwet L-77 to 0.02%, and 3 ml of bacterial suspension/each red pepper was inoculated by foliar application. To create optimal disease development, the red pepper plants were incubated at 25°C for 24 h in a dew chamber without light (vs705D Vision Scientific Co., Seoul, South Korea), and then the plants were transferred to a greenhouse with a cycle of 16 h light and 8 h dark at 28°C with 70–80% relative humidity. Disease incidences of symptoms were scored at one week after pathogen inoculation. Three independent experiments were performed with 15 plants per treatments. The tobacco

plants cv. Xanthi nc were inoculated with virus obtained from systemically infected Samsun cultivar leaves, which were ground in 50 mM potassium phosphate as described previously (Park et al., 2011). However, plants pre-treated with the chemical fungicides were not protected from these pathogenic challenges (data not shown). Our results suggest that foliar application of trifloxystrobin SC and azoxystrobin SC increased growth and induced tolerance against cold, freezing, drought, and high temperature in red pepper plants under the conditions used for the assay. However, there was no protection for a viral or bacterial caused disease, unlike the published reports for protection against fungi or against the abiotic stress of increased salinity (data not shown).

We are investigating whether trifloxystrobin SC causes stomatal closure as has been observed with biotic effectors resulting in induced drought tolerance (Cho et al., 2008). We are examining alterations in compounds and enzymes

Fig. 4. Effect of trifloxystrobin suspension concentrates (SC) on relative water content in red pepper plants under high temperature stress conditions. Three-week-old pepper seedlings grown in pots were pre-treated with various concentrations of trifloxystrobin SC, azoxystrobin SC, or water as a negative control. Five days after the application, the red pepper plants were exposed for 1 day to 42 °C, and relative water content of the leaves was measured at two days after returning to room temperature. Each bar represents the mean $\pm$ standard deviation of at least five leaflets randomly cut from 15 plants/treatment with three independent experiments. Bars with different letter superscripts are statistically different at $P < 0.05$.

associated with drought stress in trifloxystrobin SC-treated plants and drought treated plants. Other chemicals, including ketoconazole, propiconazole, imidacloprid, and azoxystrobin increase production of the antioxidant enzymes, superoxide dismutase, catalase, and peroxidases, and antioxidant compounds such as ascorbic acid and α -tocopherol contents in plants (Abdul Jaleel et al., 2007c; 2008; Gonias et al., 2008; Munne-Bosch and Penuelas, 2003; Wu and Tidemann, 2001). It is thought that systemic application of BABA induces systemic tolerance to abiotic stresses involves priming effects by expression of basal defense responses through the abscisic acid pathway (Ton et al., 2005). The chemical fungicide trizole, which mediates induction of systemic tolerance against abiotic stresses, is associated with altered plant disease-defense transcripts and modified levels of ABA, and the triazole-treated plants acquired systemic tolerance and resistance against drought and plant diseases (Ronchi et al., 1997).

In summary we have provided evidence that trifloxystrobin SC, which is a broad spectrum fungicide, also activates systemic protection to several abiotic stresses in red pepper plants such as drought, low or high temperatures and freezing injury but not salinity.

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