



## Research Paper

## Effects of the fungicides azoxystrobin, pyraclostrobin and boscalid on the physiology of Japanese cucumber



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## ABSTRACT

Strobilurins are fungicides with beneficial physiological effects on crop yield due to promotion of net carbon assimilation, nitrate reductase enzyme activity, stress tolerance and hormonal balance. The carboxamides complement the action of the strobilurins when applied alternately or together. This study aimed to evaluate the effect of application of pyraclostrobin, azoxystrobin and boscalid on grafted and ungrafted Japanese cucumber (*Cucumis sativus* L.) in order to analyze the effects of these fungicides on plant physiology and increased fruit production. The experimental design was completely randomized in a  $2 \times 5$  factorial scheme, with grafted and ungrafted cucumber plants and 5 fungicide treatments: control; azoxystrobin 60 g a.i ha<sup>-1</sup>; boscalid 50 g a.i ha<sup>-1</sup>; pyraclostrobin 50 g a.i ha<sup>-1</sup>; and boscalid 100 g a.i ha<sup>-1</sup> + pyraclostrobin 50 g a.i ha<sup>-1</sup>, applied 18 days after transplanting and then at intervals of seven days. The effect of the treatments was evaluated based on the average yield and fruit mass, in addition to observations of gas exchange, lipid peroxidation, and the activities of peroxidase, superoxide dismutase, catalase and nitrate reductase. Based on the results obtained, it was concluded that the fungicide treatments resulted in different responses between the grafted and ungrafted plants. The physiological benefits were more evident in the grafted plants treated with pyraclostrobin or boscalid alone, and these benefits manifested in terms of fruit production and increased the activity of the antioxidative system, thereby reducing stress. The higher productivity probably occurred due to the better physiological performance of these plants, mainly at the beginning of development, presenting greater activity of the enzyme nitrate reductase, in addition to the higher net CO<sub>2</sub> assimilation and carboxylation efficiency.

## 1. Introduction

Until recently, disease control was the only purpose of fungicide use; however, the physiological benefits of strobilurins opened a new concept for the use of these products (Venancio et al., 2003). Due to the large capacity of the plant to absorb them, these fungicides have positive physiological effects on the yields of crops, causing alterations in metabolism and growth (Koehle et al., 2002). This effect was observed even without any alterations caused by pathogenic fungi; the plants treated with these substances showed greater vigor and higher yield than untreated plants. The carboxamides have also been included in this group of fungicides with positive physiological effects, but because they have spent less time on the market, there is little information or experimental results regarding this family.

The physiological effect of strobilurins results from the net photosynthesis increase due to the temporary reduction of plant respiration, which causes less carbon loss, thereby generating more energy for the plant. In addition, strobilurins increase the activity of nitrate reductase and antioxidant enzymes, which leads to greater stress tolerance; additionally, increased synthesis of indoleacetic acid (IAA), isopentenyl adenine (I6-ADE) and abscisic acid (ABA) and reduced ethylene production leads to a better hormonal balance, delayed senescence and prolonged photosynthetic activity, the so-called “green effect” (Bartlett et al., 2002; Ypema and Gold, 1999; Zhang et al., 2010).

Most of the results in the literature have been obtained in experiments on field crops, such as soybean (Fagan et al., 2010; Joshi et al., 2014; Soares et al., 2011), corn (Barbosa et al., 2011; Blandino et al., 2012), wheat (Grossmann and Retzlaff, 1997; Inagaki et al., 2009;

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Ishikawa et al., 2012; Koehle et al., 2002; Ypema and Gold, 1999), bean (Jadoski et al., 2015; Kozłowski et al., 2009) and barley (Jabs et al., 2002), and there are few reports on the physiological effects of these fungicides on vegetables, although they are also used preventively in this group, mainly in the Solanaceae and Cucurbitaceae families. Studies on the physiological changes in the plant are scarce, there is much controversy on this subject, and there few data on the effects on disease-free plants.

The Japanese cucumber (*Cucumis sativus* L.) is among the crops that are most widely cultivated in a protected environment. The use of this technique allows year-round production of fruits with excellent commercial quality, reducing losses and increasing productivity (Mohammadi and Omid, 2010). However, this practice causes problems such as increased incidence of diseases and nematodes, requiring the use of grafting in resistant plants for preventive purposes to control diseases and improve the absorption of nutrients (Lee and Oda, 2010).

Agricultural practices aim to increase productivity and the quality of the final product, so it is increasingly important to understand the physiological changes that occur after the application of these important groups of fungicides. The aim of this study was to evaluate the effect of the application of pyraclostrobin, azoxystrobin and boscalid on grafted and ungrafted Japanese cucumber plants in order to analyze the effects of these fungicides on plant physiology, as well as on the increase of fruit production.

## 2. Material and methods

### 2.1. Plant material and application of fungicides

The experimental area was located at the Faculdade de Ciências Agrônomicas (FCA), Universidade Estadual Paulista (UNESP), located in São Manuel city, São Paulo state, Brazil. The study site is located at 22° 44' S latitude, 47° 34' W longitude, and an altitude of 750 m. The climate is humid subtropical mesothermic with drought conditions in the winter season. We used an arc-type greenhouse with the following characteristics: 30 m length, 7 m width and 3 m height, covered with low-density polyethylene film (150 µm) with the lateral sides covered with a 75% shade cloth.

The experiment was conducted with grafted and ungrafted Japanese cucumber plants (*Cucumis sativus* L.). The Japanese cucumber hybrid 'Taisho' (scion) was grafted onto the pumpkin hybrid 'Excite Ikki' (root-stock) using the tongue approach grafting method. To ensure that both hypocotyl diameters were similar, allowing proper grafting, the pumpkins were sown 4 days before the cucumbers. The grafting was performed 10 days after sowing the cucumbers, and the plants were transplanted into pots 4 days after grafting. Thereafter, the seedlings were kept in a moist chamber until they were suitable for transplantation.

A spacing of 1.0 × 0.5 m was used between seedlings. The seedlings were guided conducted with one stem oriented vertically to avoid damage to fruit production or quality. We removed all shoots and eliminated all buds and flowers from the 1st node through the 5th node, allowing the side branches to grow starting from the 6th node; the side branches were topped and tailed after the 3rd internode.

The experimental design was completely randomized in a 2 × 5 factorial scheme, with grafted and ungrafted cucumber plants and 5 treatments with fungicides: control; azoxystrobin 60 g a.i ha<sup>-1</sup>; boscalid 50 g a.i ha<sup>-1</sup>; pyraclostrobin 50 g a.i ha<sup>-1</sup>; and boscalid 100 g a.i ha<sup>-1</sup> + pyraclostrobin 50 g a.i ha<sup>-1</sup>, applied 18 days after transplanting and then at intervals of seven days.

The azoxystrobin source (strobilurin) was the product Amistar® containing 500 g kg<sup>-1</sup> a.i.; for boscalid, the product was Cantus®, containing 500 g kg<sup>-1</sup> a.i.; for pyraclostrobin (strobilurin), the product used was Comet®, containing 250 g L<sup>-1</sup> i.a.; and for the mixture of boscalid and pyraclostrobin, the product used was Bellis®, containing 200 g kg<sup>-1</sup> boscalid and 100 g kg<sup>-1</sup> pyraclostrobin.

The fungicide applications were carried out via foliar spraying using a manual pressurized CO<sub>2</sub> sprayer with 0.3 kg f cm<sup>-2</sup> with conical nozzles and using a plastic curtain between treatments to avoid drift. Healthy plants were used so that only the physiological effect, rather than the antifungal effect, could be observed.

The first application of the treatments was carried out 18 days after transplanting (DAT), when the seedlings had 6 completely expanded leaves, and subsequent applications were at seven days intervals, with four replicates and six plants per plot, assuming four useful plants.

Physiological and biochemical evaluations were performed at the beginning of the harvest (35 DAT), the peak of the harvest (57 DAT) and the end of the harvest (80 DAT); at each time point, the second fully expanded leaf that was healthy and without signs of senescence was selected and standardized.

### 2.2. Gas exchange

Gas exchange was measured with an infrared CO<sub>2</sub> and water vapor analyzer (LI-6400, Li-Cor Inc., Lincoln NE, USA). The measurements were performed from 9:00 am until 11:00 am on a sunny day. The net assimilation rate ( $A_{net}$ , µmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>), transpiration ( $E$ , mmol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>) and stomatal conductance ( $g_s$ , mol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>) were measured. The water use efficiency ( $WUE$ , µmol CO<sub>2</sub> (mmol H<sub>2</sub>O)<sup>-1</sup>) was determined by the relationship between net assimilation rate and transpiration, and the apparent carboxylation efficiency ( $A_{net}/C_i$ ) was determined by the relationship between the net assimilation rate and the intercellular CO<sub>2</sub> concentration ( $C_i$ , µmol CO<sub>2</sub> mol<sup>-1</sup> air).

To ensure that the experimental conditions were consistent, the PPFD was standardized through the use of a light-emitting diode coupled to a photosynthesis chamber, and the light-emitting diode emitted 1500 µmol m<sup>-2</sup> s<sup>-1</sup>, as this is the saturating luminosity, according to a light curve previously performed (Amaro et al., 2014). The reference CO<sub>2</sub> concentration that was used during the evaluation was the ambient value, which ranged from 380 to 400 µmol mol<sup>-1</sup> of air.

### 2.3. Biochemical analyses

The leaf blades were collected and placed in plastic bags, wrapped in aluminum and immediately frozen in liquid nitrogen to stop all metabolic reactions. The leaf blade samples were stored in a -80 °C freezer for further analysis.

#### 2.3.1. Measurement of nitrate reductase activity

For the determination of the activity of the nitrate reductase enzyme (EC 1.6.6.1), leaf blades (1 g) were sliced and placed in a dark vial containing 8 mL of potassium phosphate buffer (0.1 M, pH 7.0) and KNO<sub>3</sub> (0.02 M). Thereafter, the vials were incubated for one hour at 37 °C in the absence of light. After this period, 1 mL of sulfanilamide solution (1%) and 1 mL of N-Naphthyl solution (0.02%) were added and then incubated at 37 °C in the dark for 5 min. After this period, the nitrate reductase activity was determined spectrophotometrically at 540 nm. A nitrite solution was used to construct the standard curve (Streeter and Bosler, 1972).

#### 2.3.2. Measurement of antioxidant enzyme activities and lipid peroxidation

For enzyme extracts, the leaf blades (300 mg) were pulverized in liquid nitrogen and homogenized in 4 mL of pre-cooled potassium phosphate buffer (0.1 M, pH 6.8) and 200 mg PVP. The homogenates were centrifuged at 10,000 × g for 10 min at 4 °C, and the resulting supernatants were used for enzyme assays (Kar and Mishra, 1976). The soluble protein content was determined using casein as a standard (Bradford, 1976). The supernatant from the extraction was used to determine the activities of the enzymes superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7) and catalase (CAT, EC 1.11.1.6).

The SOD activity was determined by spectrophotometrically

measuring the photochemical inhibition of nitroblue tetrazolium (NBT) at 560 nm (Peixoto et al., 1999). One unit of SOD activity (U) was defined as the amount of enzyme required to inhibit the NBT photo-reduction by 50%, and the specific activity of the enzyme was expressed as  $\text{U mg}^{-1}$  protein.

POD activity was determined in a spectrophotometer by measuring the formation of purpurogallin at 430 nm (Teisseire and Guy, 2000), and the activity was expressed in  $\mu\text{mol}$  of purpurogallin  $\text{min}^{-1} \text{mg}^{-1}$  protein. The activity of CAT was assayed by monitoring  $\text{H}_2\text{O}_2$  consumption at 240 nm for 80 s (Peixoto et al., 1999), and the activity was expressed in  $\text{nmol}$  of  $\text{H}_2\text{O}_2$  consumed  $\text{min}^{-1} \text{mg}^{-1}$  protein.

For the lipid peroxidation assay, the leaf blades (200–400 mg) were pulverized in liquid nitrogen and homogenized in 5 mL of solution containing 0.25% thiobarbituric acid (TBA) and 10% trichloroacetic acid (TCA). The extracted solution was incubated in a water bath at 90 °C for 60 min. After cooling, the solution was centrifuged at 10,000  $\times g$  for 15 min at room temperature (25 °C). The supernatant collected from each sample was subjected to spectrophotometric absorbance readings at 560 and 600 nm. For the calculations, the molar extinction coefficient of malondialdehyde ( $155 \text{ mmol L}^{-1} \text{cm}^{-1}$ ) was used (Rama Devi and Prasad, 1998).

## 2.4. Fruit production

Fruit harvesting was performed daily starting at 31 DAT and ending at 80 DAT. We evaluated the number of fruits  $\text{m}^{-2}$ , yield ( $\text{kg m}^{-2}$ ) and average weight ( $\text{g fruit}^{-1}$ ) when they were 20–25 cm long and 2.0–3.0 cm in diameter.

## 2.5. Statistical analyses

The statistical package used for the data analysis was SAS 9.2 (SAS Institute Inc., Cary, NC). The Levene test was used to verify the homogeneity of variances of the treatments. The data were examined using analysis of variance (ANOVA). The experimental design was completely randomized in a  $2 \times 5$  factorial scheme, with grafted and ungrafted cucumber plants and 5 treatments with fungicides. The means were compared using Tukey test ( $P \leq 0.05$ ).

## 3. Results

The Levene test showed that the variances were homogeneous among the treatments and grafting for all variables analyzed.

### 3.1. Gas exchange

The significant interaction treatments  $\times$  grafting was detected for gas exchanges ( $P < 0.001$ ), except for  $g_s$  ( $P > 0.05$ ). At the beginning of fruit production (35 DAT), grafted plants treated with pyraclostrobin and boscalid alone presented the highest  $A_{net}$  and  $A_{net}/C_i$  despite there being no statistically significant differences between their  $WUE$  and  $g_s$  and those of control plants (Table 1). However, at the peak (57 DAT) and final fruit production (73 DAT), there was an increase in the gas exchange rates ( $A_{net}$ ,  $WUE$  and  $A_{net}/C_i$ ) of the control plants. The gas exchange rates of the control plants remained higher than those of the plants treated with the fungicides but did not differ from the plants treated with pyraclostrobin and boscalid alone, whereas they did not decrease in relation to the previous evaluation. Plants treated with azoxystrobin had the lowest rates of gas exchange ( $A_{net}$  and  $A_{net}/C_i$ ) throughout the experiment (Table 1).

Ungrafted plants treated with azoxystrobin had the lowest rates of gas exchange ( $A_{net}$ ,  $WUE$  and  $A_{net}/C_i$ ) at the beginning of fruit production (35 DAT); however, the gas exchange rates of the plants receiving the other treatments with did not differ from those of the control (Table 1). At the peak of fruit production (57 DAT), all fungicide treatments efficiently increased the gas exchange rates ( $A_{net}$ ,  $WUE$  and

$A_{net}/C_i$ ) of ungrafted plants, and the strobilurin (azoxystrobin and pyraclostrobin) treatments were the most efficient. At the end of fruit production (73 DAT), plants treated with azoxystrobin or the mixture of pyraclostrobin and boscalid maintained higher  $A_{net}$  and  $A_{net}/C_i$  but these values did not differ statistically from those of control plants (Table 1).

In general, at 35 DAT, the application of azoxystrobin, boscalid and pyraclostrobin increased the gas exchange of the grafted plants; however, this response was not maintained in the other time points (Table 1).

### 3.2. Fungicide effects on nitrate reductase activity

The significant interaction treatments  $\times$  grafting was detected for nitrate reductase activity ( $P < 0.001$ ). At the beginning of fruit production (35 DAT), all fungicides efficiently increased the activity of the nitrate reductase enzyme in the grafted plants in relation to the control (Table 2), and the plants treated with pyraclostrobin, azoxystrobin or boscalid alone presented the highest values. The ungrafted plants treated with pyraclostrobin alone showed the highest activity of the nitrate reductase enzyme. Plants treated with the mixture of pyraclostrobin and boscalid showed the second highest activity of the nitrate reductase enzyme, but this activity did not differ from that in plants treated with azoxystrobin or boscalid alone (Table 2). It was also observed that at 35 DAT, the application of azoxystrobin or boscalid alone increased nitrate reductase activity in the grafted plants relative to that in the ungrafted plants.

At the peak of fruit production (57 DAT), ungrafted plants treated with pyraclostrobin alone continued to show the highest activity of the nitrate reductase enzyme in, but this activity did not differ from that in plants treated with azoxystrobin (Table 2). In the grafted plants, the effects of treatments with fungicides did not differ from those of the control. At the end of the harvest (73 DAT), grafted plants treated with the fungicides showed less activity of the enzyme nitrate reductase. However, ungrafted plants treated with strobilurins (pyraclostrobin alone and azoxystrobin) remained similar to the controls (Table 2).

### 3.3. Effects of fungicides on antioxidant enzyme activity and lipid peroxidation

The significant interaction treatments  $\times$  grafting was detected for antioxidant enzyme activity and lipid peroxidation ( $P < 0.001$ ). The ungrafted plants treated with the pyraclostrobin and boscalid mixture showed an increase in the activity of the antioxidative system. The ungrafted plants presented greater SOD and CAT activities and lower lipid peroxidation than the control plants at 35 (Fig. 1) and 73 DAT (Fig. 3), evidencing their role as a protector against stress. In the grafted plants, this mixture of fungicides was also efficient at combating oxidative stress, as treatment with the mixture presented the lowest level of lipid peroxidation at 35 DAT, as well as higher POD activity in relation to the control despite the activity of SOD being lower (Fig. 1).

The ungrafted plants treated with azoxystrobin presented the highest level of lipid peroxidation at 35 DAT, 50% higher than in the control treatment. The ungrafted plants also showed high SOD activity but low CAT activity, which is inefficient at this stage in the detoxification of hydrogen peroxide, thus failing to combat stress and explaining the higher lipid peroxidation levels (Fig. 1); however, this finding was not replicated for the other enzymatic collections, because the plants treated with this fungicide showed increased SOD activity in relation to the control at 57 (Fig. 2) and 73 DAT (Fig. 3) and increased POD and CAT at 57 DAT (Fig. 2), showing that the plants had recovered from the stress at the previous evaluation. These findings suggest that enzyme activity was still settling at that time, maintaining low lipid peroxidation levels.

In the grafted plants, among all treatments, azoxystrobin treatment resulted in the lowest activity of antioxidant enzymes (SOD, POD and

**Table 1**

Net CO<sub>2</sub> assimilation rate ( $A_{\text{net}}$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), transpiration ( $E$ ,  $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$ ), stomatal conductance ( $g_s$ ,  $\text{mol m}^{-2} \text{s}^{-1}$ ), water use efficiency ( $WUE$ ,  $\mu\text{mol CO}_2 (\text{mol H}_2\text{O})^{-1}$ ) and carboxylation efficiency ( $A_{\text{net}}/C_i$ ) in ungrafted and grafted Japanese cucumber (*C. sativus*) leaves sprayed with different fungicides.

35 DAT										
Treatments	$A_{\text{net}}$		$E$		$g_s$		$WUE$		$A_{\text{net}}/C_i$	
	Ungrafted	Grafted	Ungrafted	Grafted	Ungrafted	Grafted	Ungrafted	Grafted	Ungrafted	Grafted
Control	22.13 aA	22.27 bA	10.80 aA	9.51 bcA	0.8047 aA	0.8584 abA	2.05 aB	2.43 abA	0.0724 aA	0.0728 bA
AZB	15.62 bB	18.48 cA	10.24 aA	8.80 cB	0.6866 aA	0.6415 bA	1.53 bB	2.09 bcA	0.0484 bB	0.0622 cA
BOS	22.03 aB	27.50 aA	10.14 aA	11.13 abA	0.8383 aA	1.1407 aA	2.17 aB	2.47 aA	0.0718 aB	0.0918 aA
PIR	22.87 aB	27.17 aA	10.23 aB	11.77 aA	0.7250 aA	1.0107 abA	2.26 aA	2.31 abcA	0.0781 aB	0.0910 aA
BOS + PIR	23.33 aA	21.10 bcB	10.86 aA	10.51 abcA	0.8477 aA	0.6415 bA	2.15 aA	2.03 cA	0.0775 aA	0.0714 bcA
F value										
Treatments	37.59**		2.82*		3.01 ns		10.80**		43.00**	
Grafting	20.26**		0.128 ns		0.754 ns		16.55**		27.56**	
Treatments x Grafting	9.008**		3.80*		2.150 ns		4.52**		9.37**	
C.V. (%)	6.64		9.32		27.62		8.38		6.7	
57 DAT										
Treatments	$A_{\text{net}}$		$E$		$g_s$		$WUE$		$A_{\text{net}}/C_i$	
	Ungrafted	Grafted	Ungrafted	Grafted	Ungrafted	Grafted	Ungrafted	Grafted	Ungrafted	Grafted
Control	23.07 bB	30.10 aA	11.00 aA	10.25 abA	0.9667 aA	1.0317 aA	2.00 bB	2.94 aA	0.0780 bB	0.1095 aA
AZB	28.00 aA	25.30 bcB	10.25 aA	11.03 aA	1.0900 aA	0.9390 aA	2.74 aA	2.30 bB	0.0983 aA	0.0878 bA
BOS	26.42 aA	27.40 abcA	10.57 aA	9.54 bB	1.0892 aA	0.8870 aA	2.51 abB	2.88 aA	0.0906 abA	0.0998 abA
PIR	26.97 aA	27.80 abA	10.52 aA	10.30 abA	0.9192 aA	1.126 aA	2.57 aA	2.73 abA	0.0974 aA	0.0971 abA
BOS + PIR	27.15 aA	24.40 cB	10.04 aA	10.43 abA	1.1310 aA	0.7757 aB	2.71 aA	2.35 bB	0.0951 aA	0.0870 bA
F value										
Treatments	1.24 ns		1.32 ns		0.13 ns		1.21 ns		0.76 ns	
Grafting	2.05 ns		0.72 ns		1.63 ns		2.94 ns		3.39 ns	
Treatments x Grafting	14.23**		3.00*		2.13 ns		12.18**		10.18**	
C.V. (%)	5.61		5.96		21.72		8.36		8	
73 DAT										
Treatments	$A_{\text{net}}$		$E$		$g_s$		$WUE$		$A_{\text{net}}/C_i$	
	Ungrafted	Grafted	Ungrafted	Grafted	Ungrafted	Grafted	Ungrafted	Grafted	Ungrafted	Grafted
Control	22.34 abA	24.07 aA	10.98 bA	11.20 aA	0.6304 aA	0.7493 aA	2.03 aA	1.16 aA	0.0818 abA	0.0886 aA
AZB	25.32 aA	17.80 bB	11.46 abA	9.58 bB	0.8449 aA	0.7503 aA	2.21 aA	2.10 aB	0.0927 aA	0.0638 bB
BOS	23.13 abA	23.37 aA	12.07 aA	11.05 aB	1.0938 aA	0.6725 aB	1.92 aA	2.11 aA	0.0789 abA	0.0864 aA
PIR	20.13 bA	23.67 aB	10.61 bA	11.00 aA	0.9120 aA	0.6673 aA	1.89 aB	2.15 aA	0.0719 bB	0.0870 aA
BOS + PIR	24.50 aA	24.77 aA	11.37 abA	11.82 aA	0.6897 aA	0.7930 aA	2.16 aA	1.86 aA	0.0914 aA	0.0899 aA
F value										
Treatments	2.87*		8.36**		0.80 ns		0.71 ns		2.27 ns	
Grafting	0.29 ns		6.35*		2.24 ns		0.39 ns		0.01 ns	
Treatments x Grafting	8.52**		10.22**		2.06 ns		4.40**		6.75**	
C.V. (%)	8.95		4.13		29.15		8.11		11.17	

AZB – azoxistrobin 60 g a.i ha<sup>-1</sup>; BOS – boscalid 50 g a.i ha<sup>-1</sup>; PIR – piraclostrobin 50 g a.i ha<sup>-1</sup>; BOS + PIR – boscalid 100 g a.i ha<sup>-1</sup> + piraclostrobin 50 g a.i ha<sup>-1</sup>; DAT – days after transplant. Values followed by the same letter do not differ significantly according to Tukey's test ( $n = 4$ ); lower case letters in the column and capital letters in the line. \*Significant at 1% probability ( $P \leq 0.01$ ); \*significant at 5% probability ( $P \leq 0.05$ ); and ns denotes not significant ( $P > 0.05$ ).

CAT) at the beginning and at the end of fruit production (35 and 73 DAT). However, the level of lipid peroxidation remained low, suggesting that enzyme activity had been higher at some earlier time, combating ROS and resulting in a low level of oxidative stress, but at the time of collection, the enzyme activities had already decreased (Figs. 2 and 3). At the peak of production (57 DAT), the activity of SOD, POD and CAT was among the highest (Fig. 2), and as the levels of lipoperoxides were relatively low, we can conclude that oxidative stress had been controlled.

At the end of production (73 DAT), the application of fungicides in the grafted plants decreased the lipid peroxidation levels in relation to those in the control plants. for the exception was the plants treated with pyraclostrobin, which showed lower activities of the antioxidant enzymes SOD, POD and CAT and led to one of the highest lipid peroxidation rates, which was similar to that of the control (Fig. 3).

### 3.4. Effects of fungicide on fruit production

The significant interaction treatments x grafting was detected for fruit production ( $P < 0.001$ ), except for average weight ( $P > 0.05$ ). In relation to the measures of production, the number of fruits m<sup>-2</sup> and productivity (kg m<sup>-2</sup>), in the ungrafted plants, the application of the mixture of pyraclostrobin with boscalid was the only treatment that increased fruit production (Table 3), resulting in 17% more fruit and 14% more productivity; however, the results were not significantly different from those of the control plants. In addition, ungrafted plants did not present significant differences in average fruit mass.

In the grafted plants, all treatments with fungicides increased the number of fruits and the productivity (kg m<sup>-2</sup>) (Table 3). The plants treated with pyraclostrobin alone produced 64% more fruits than the control and were 60% more productive; however, the results were not

**Table 2**Nitrate reductase activity (NR) in ungrafted and grafted Japanese cucumber (*C. sativus*) leaves sprayed with different fungicides.

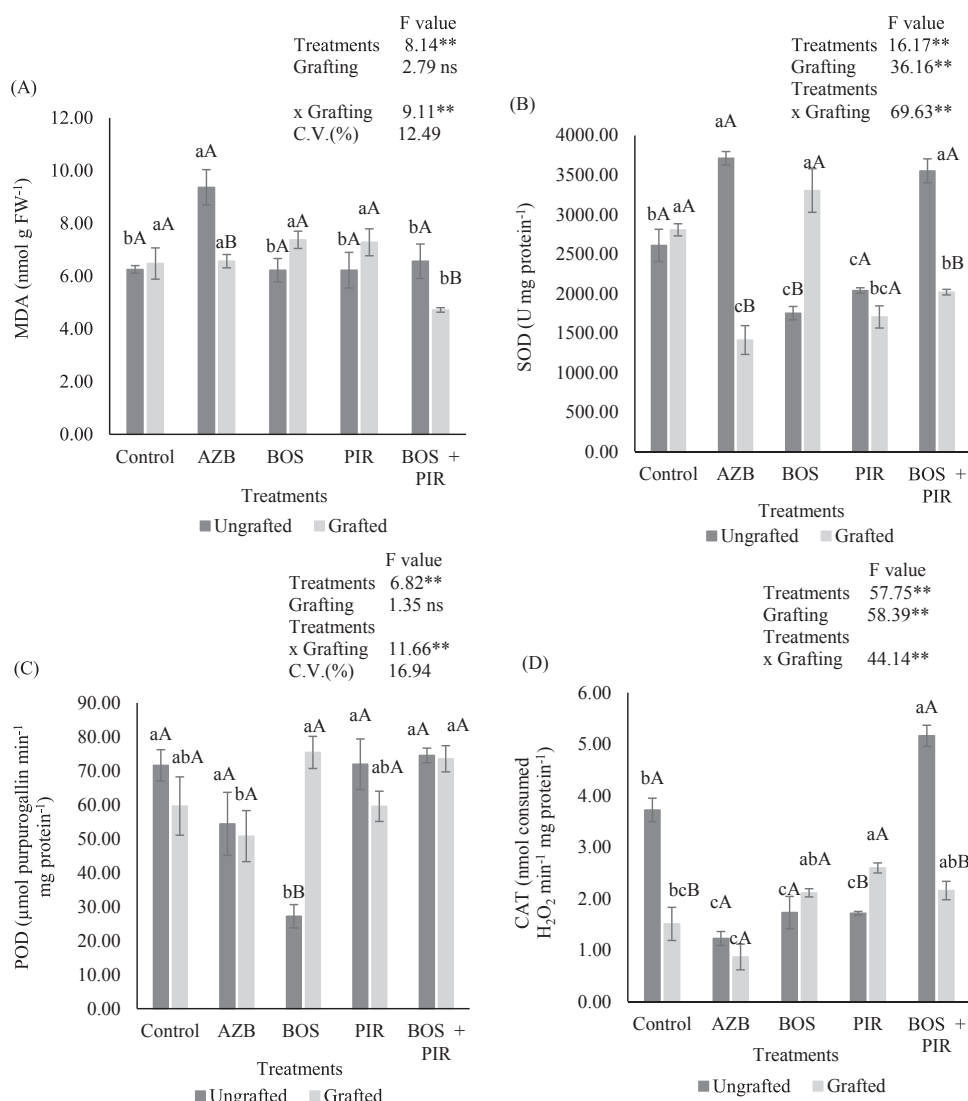
Treatments	35 DAT		56 DAT		73 DAT	
	Ungrafted	Grafted	Ungrafted	Grafted	Ungrafted	Grafted
Control	55.95 cA	61.20 cA	51.40 bcA	50.00 aA	118.39 aB	164.50 aA
AZB	62.68 bcB	115.59 aA	58.64 abA	49.91 aB	95.40 abB	154.92 abA
BOS	70.59 bcB	107.46 aA	42.53 cA	48.25 abA	64.82 bA	63.42 cA
PIR	109.87 aA	111.12 aA	67.51 aA	44.40 abB	123.41 aA	122.83 bA
BOS + PIR	74.43 bA	78.94 bA	54.32 bA	38.62 cB	68.91 bA	68.38 cA
F value						
Treatments	48.83**		7.14**		32.38**	
Grafting	68.42**		30.90**		13.62**	
Treatments x Grafting	18.32**		10.71**		5.68**	
C.V. (%)	9.09		9.73		16.91	

AZB – azoxistrobin 60 g a.i ha<sup>-1</sup>; BOS – boscalid 50 g a.i ha<sup>-1</sup>; PIR – piraclostrobin 50 g a.i ha<sup>-1</sup>; BOS + PIR – boscalid 100 g a.i ha<sup>-1</sup> + piraclostrobin 50 g a.i ha<sup>-1</sup>; DAT – days after transplant. Values followed by the same letter do not differ significantly according to Tukey's test ( $n = 4$ ); lower case letters in the column and capital letters in the line. \*\*Significant at 1% probability ( $P \leq 0.01$ ); \*significant at 5% probability ( $P \leq 0.05$ ); and ns denotes not significant ( $P > 0.05$ ).

significantly different from those of the plants treated with boscalid alone. Plants treated with boscalid had 40% more fruits and we 34% more productive.

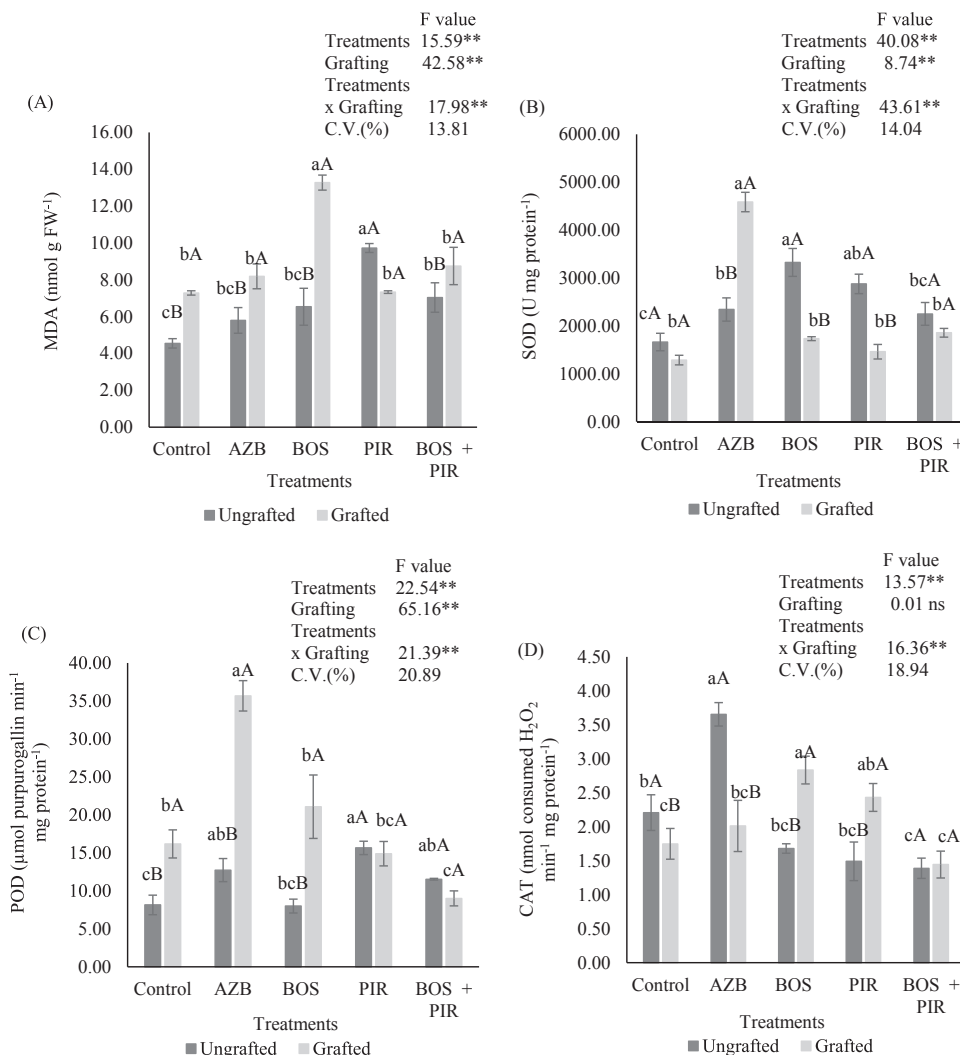
The mixture of pyraclostrobin and boscalid in the grafted plants

resulted in 17% more fruits and 11% greater productivity than in the control plants, but there were no significant differences; the increases were similar to those in ungrafted plants. This result indicates that the efficiency had not diminished; rather, the efficiency of the other



**Fig. 1.** (A) Lipid peroxidation (MDA), (B) superoxide dismutase activity (SOD), (C) peroxidase activity (POD) and (D) catalase activity (CAT) in ungrafted and grafted Japanese cucumber (*C. sativus*) leaves sprayed with different fungicides, at 35 days after transplant. Columns represent the mean and the error bars represent standard error ( $n = 4$ ). Lowercase letters indicate significance differences according to Tukey test among the treatments. Different capital letters indicate significance differences according to Tukey test among presence of grafting. \*\*Significant at 1% probability ( $P \leq 0.01$ ); \*significant at 5% probability ( $P \leq 0.05$ ); and ns denotes not significant ( $P > 0.05$ ). AZB – azoxistrobin 60 g a.i ha<sup>-1</sup>; BOS – boscalid 50 g a.i ha<sup>-1</sup>; PIR – piraclostrobin 50 g a.i ha<sup>-1</sup>; BOS + PIR – boscalid 100 g a.i ha<sup>-1</sup> + piraclostrobin 50 g a.i ha<sup>-1</sup>.





**Fig. 2.** (A) Lipid peroxidation (MDA), (B) superoxide dismutase activity (SOD), (C) peroxidase activity (POD) and (D) catalase activity (CAT) in ungrafted and grafted Japanese cucumber (*C. sativus*) leaves sprayed with different fungicides, at 57 days after transplant. Columns represent the mean and the error bars represent standard error ( $n = 4$ ). Lowercase letters indicate significance differences according to Tukey test among the treatments. Different capital letters indicate significance differences according to Tukey test among presence of grafting. \*\*Significant at 1% probability ( $P \leq 0.01$ ); \*significant at 5% probability ( $P \leq 0.05$ ); and ns denotes not significant ( $P > 0.05$ ). AZB – azoxystrobin 60 g a.i ha<sup>-1</sup>; BOS – boscalid 50 g a.i ha<sup>-1</sup>; PIR – piraclostrobin 50 g a.i ha<sup>-1</sup>; BOS + PIR – boscalid 100 g a.i ha<sup>-1</sup> + piraclostrobin 50 g a.i ha<sup>-1</sup>.

fungicides had increased. However, the plants treated with azoxystrobin presented heavier fruits although they did not differ from those of the control plants (Table 3).

Regarding the absence or presence of grafting, some differences were also found. The ungrafted plants controlled and treated with the mixture of pyraclostrobin and boscalid showed higher productivity and a greater number of fruits than the grafted plants (Table 3). However, when the grafted plants were treated with pyraclostrobin alone, this result was opposite, with the grafted plants showing greater fruit number and productivity.

#### 4. Discussion

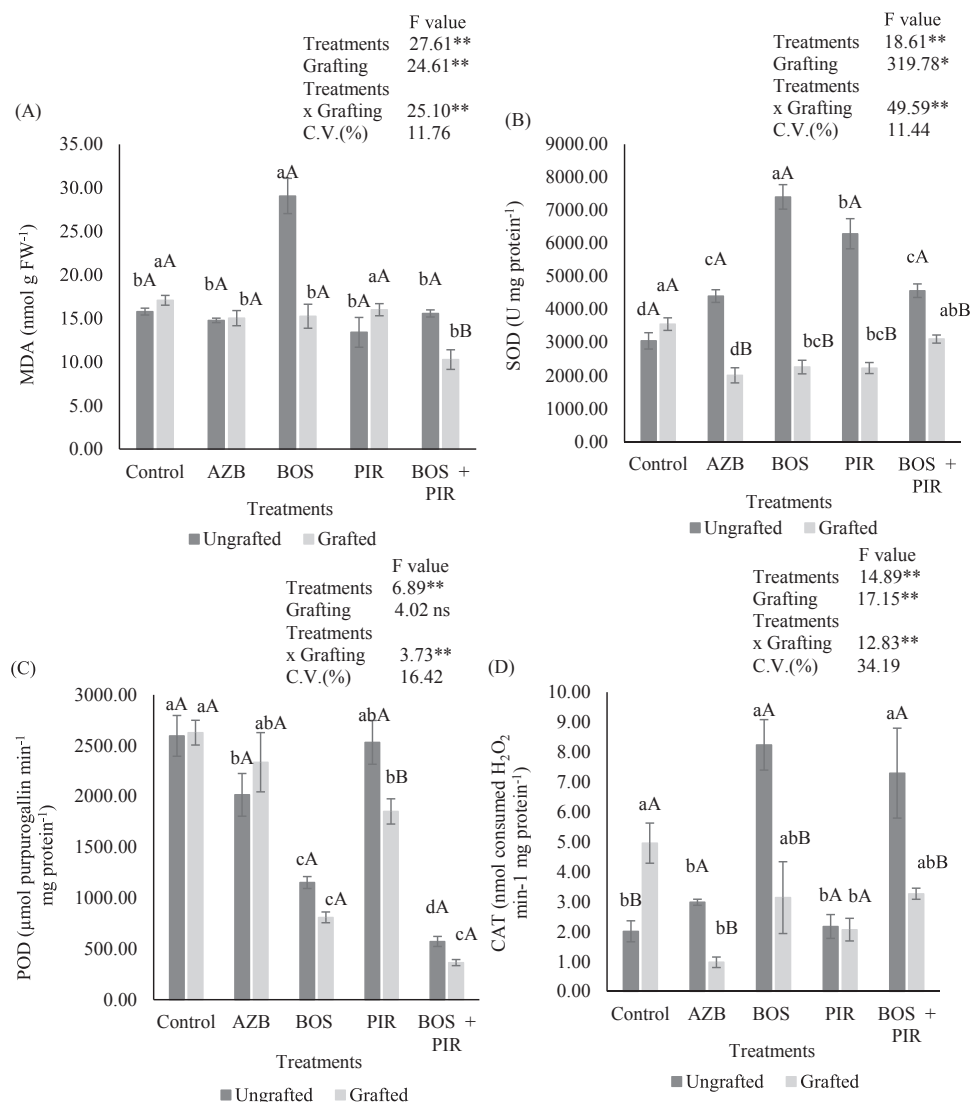
In the present study, it was verified that the different strobilurins tested exert different physiological effects on the plants to which they are applied, possibly due to their different formulations. While the application of pyraclostrobin resulted in an improvement in the physiological performance of grafted cucumber plants, treatment with azoxystrobin had no such effect. Similar results were found in the literature, where it was reported that the application of azoxystrobin also decreased the rate of net CO<sub>2</sub> assimilation, transpiration rate, stomatal conductance and internal carbon concentration in the substomatal chamber in wheat (Nason et al., 2007), barley (Nason et al., 2007), soybean (Nason et al., 2007) and rice (Debona et al., 2016). One possible explanation for these results is that the reduction of ATP production caused by the blockade of electron transport in the cytochrome

bc<sub>1</sub> complex may, instead of increasing the rate of CO<sub>2</sub> assimilation by temporarily reducing respiration, cause the normal functioning of metabolism in guard cells, reducing turgor and consequently stomatal opening, as the osmotic potential of guard cells is limited by the production of ATP (Nason et al., 2007). The same authors verified that the ratio of *Fv/Fm* is also decreased in soybean plants, which indicates that photosynthesis is directly inhibited, perhaps independently of what might have occurred in the stomata. One possible explanation is that these strobilurins block the transport of electrons between photosystem II I and photosystem I (PSI) by binding to the Q<sub>i</sub> site of the chloroplast *bf* cytochrome complex (Nason et al., 2007).

In this experiment, the application of fungicides to the ungrafted plants did not generally influence the levels of lipid peroxidation; they remained similar to those of the control plants, evidencing the absence of stress. The few positive physiological effects found in ungrafted plants were not sufficient to result in higher productivity.

The present study showed that fungicide treatments presented different responses between grafted and ungrafted plants. In the present work, the physiological benefits of the fungicides were more noticeable in the grafted plants, and the fungicides tested showed benefits in the production of fruits, to a greater or lesser extent, in this type of plant.

The physiology of grafted plants tends to be different from that of ungrafted plants due to graft and rootstock ratios. It is worth remembering that unlike plants that are not grafted, grafted plants undergo initial stress in the post-grafting reestablishment, since they have to adapt to the union between graft and rootstock to complete the



**Fig. 3.** (A) Lipid peroxidation (MDA), (B) superoxide dismutase activity (SOD), (C) peroxidase activity (POD) and (D) catalase activity (CAT) in ungrafted and grafted Japanese cucumber (*C. sativus*) leaves sprayed with different fungicides, at 73 days after transplant. Columns represent the mean and the error bars represent standard error ( $n = 4$ ). Lowercase letters indicate significance differences according to Tukey test among the treatments. Different capital letters indicate significance differences according to Tukey test among presence of grafting. \*\*Significant at 1% probability ( $P \leq 0.01$ ); \*significant at 5% probability ( $P \leq 0.05$ ); and ns denotes not significant ( $P > 0.05$ ). AZB – azoxistrobin 60 g a.i ha<sup>-1</sup>; BOS – boscalid 50 g a.i ha<sup>-1</sup>; PIR – piraclostrobin 50 g a.i ha<sup>-1</sup>; BOS + PIR – boscalid 100 g a.i ha<sup>-1</sup> + piraclostrobin 50 g a.i ha<sup>-1</sup>.

vascular connection (Irisarri et al., 2015; Liu et al., 2014). However, over time, the grafted plants will have more advantages than the ungrafted plants because of the vigor of the rootstock, as observed in this experiment.

Most studies of grafting suggest that changes in the scion are controlled by the rootstock through controlled uptake, synthesis, and

translocation of water, minerals, and plant hormones (Martínez-Ballesta et al., 2010). Moreover, a rootstock can improve tolerance to heavy metals and organic pollutants (Savvas et al., 2010), increases plant strength and increases resistance to abiotic stress, such as low temperatures (Ahn et al., 1999; Schwarz et al., 2010; Venema et al., 2008), drought (Rouphael et al., 2010; Schwarz et al., 2010) and

**Table 3**

Number of fruits m<sup>-2</sup>, yield and average weight in ungrafted and grafted Japanese cucumber (*C. sativus*) leaves sprayed with different fungicides.

Treatments	Number of fruits m <sup>-2</sup>		Yield (kg m <sup>-2</sup> )		Average weight (g fruit <sup>-1</sup> )	
	Ungrafted	Grafted	Ungrafted	Grafted	Ungrafted	Grafted
Control	30.76 abA	22.17 cB	3.85 abA	2.82 cB	125,27 aA	129,64 abA
AZB	24.30 bA	25.33 bcA	3.08 bB	3.77 abcA	127,03 aB	147,17 aA
BOS	26.10 bA	31.08 abA	3.23 bA	3.79 abA	123,92 aA	122,35 bA
PIR	27.56 bB	36.37 aA	3.19 bB	4.51 aA	116,17 aA	123,82 bA
BOS + PIR	36.13 aA	25.96 bcB	4.39 aA	3.13 bcB	122,03 aA	120,62 bA
F value						
Treatments	5.73**		1.80 ns		5.21**	
Grafting	0.49 ns		0.12 ns		4.65*	
Treatments x Grafting	11.00**		11.88**		2.16 ns	
C.V. (%)	12.44		13.01		6.81	

AZB – azoxistrobin 60 g a.i ha<sup>-1</sup>; BOS – boscalid 50 g a.i ha<sup>-1</sup>; PIR – piraclostrobin 50 g a.i ha<sup>-1</sup>; BOS + PIR – boscalid 100 g a.i ha<sup>-1</sup> + piraclostrobin 50 g a.i ha<sup>-1</sup>; DAT – days after transplant. Values followed by the same letter do not differ significantly according to Tukey's test ( $n = 4$ ); lower case letters in the column and capital letters in the line. \*\*Significant at 1% probability ( $P \leq 0.01$ ); \*significant at 5% probability ( $P \leq 0.05$ ); and ns denotes not significant ( $P > 0.05$ ).

salinity (Albacete et al., 2009; Colla et al., 2012), modifies genes expression levels (Baron et al., 2016) as well as controlling physiological disorders (Martínez-Ballesta et al., 2010) and improving fruit quality (Davis et al., 2008; Flores et al., 2010; Rouphael et al., 2010), thus all these factors result in higher production and increase the harvest period. As a result of this metabolic change, grafting affects photosynthetic metabolism by means of net CO<sub>2</sub> assimilation rate improvement and decrease of the maximum quantum yield of photosynthesis (Amaro et al., 2014).

The higher productivity of grafted plants treated with fungicides may have occurred due to the better physiological performance of these plants, especially at the beginning of fruit production. Similar results were observed in soybean (Soares et al., 2011) and corn (Barbosa et al., 2011), where a marked increase of nitrate reductase enzyme activity occurred at the beginning of plant development. The nitrate reductase activity changes according to the plant's life stage; thus, it has its greatest activity in growth organs when they are young since these organs require large amounts of nitrate (Larcher, 2006). In grapes (Berkelmann-Loehnertz et al., 2001), corn (Blandino et al., 2012), soybeans (Fagan et al., 2010) and beans (Jadoski et al., 2015), the application of different strobilurins also increased CO<sub>2</sub> assimilation rates.

The plants grafted and treated with pyraclostrobin or boscalid alone were those that produced the most fruits and presented higher productivity. Increases in productivity after the application of strobilurins have also been reported in tomatoes (Cantore et al., 2016), grapes (Berkelmann-Loehnertz et al., 2001; Diaz-Espejo et al., 2012), wheat (Ishikawa et al., 2012; Jørgensen and Olesen, 2002; Kildea et al., 2010; Ruske et al., 2003), corn (Barbosa et al., 2011; Blandino et al., 2012), beans (Jadoski et al., 2015; Kozłowski et al., 2009), soybeans (Fagan et al., 2010; Soares et al., 2011) and *Butterhead* lettuce (Bonasia et al., 2013). The higher production of fruits of these plants was possibly due to the greater assimilation of CO<sub>2</sub> and the better use of nitrogen.

Pyraclostrobin is member of the Q<sub>o</sub>I<sub>s</sub>, or inhibitors of the bc<sub>1</sub> complex, which inhibit the transfer of electrons in complex III of the respiratory chain, also known as the bc<sub>1</sub> cytochrome complex (Balba, 2007; Bartlett et al., 2002; Sauter et al., 1999). Thus, pyraclostrobin alters the CO<sub>2</sub> compensation point, temporarily decreasing plant respiration, which increases the rate of net CO<sub>2</sub> assimilation; it is worth noting that the bc<sub>1</sub> complex is present in all eukaryotic organisms (Grossmann and Retzlaff, 1997). This partial inhibition of the electron transport chain by strobilurins causes a decrease in cellular levels of ATP and, consequently, an increase in the concentration of protons (H<sup>+</sup>) in the cytosol, momentarily decreasing the pH (acidification). This result is due to the decrease in ATPase activity, delaying the pumping of protons from the cytosol (H<sup>+</sup>) to the apoplast and leading to increased nitrate reductase activity, as the activity of this enzyme is regulated by the pH of the cytosol (Glaab and Kaiser, 1999).

Nitrate reductase catalyzes the first nitrate uptake step from the soil and is considered key to the increase in the biomass of the plants treated with strobilurin, since this increase requires assimilation of large amounts of nitrogen, which is incorporated into organic molecules, such as amino acids and nucleotides, that are essential for plant growth (Koehle et al., 2002).

Boscalid is a succinate dehydrogenase inhibitor (SDHI) and inhibits the transport of electrons in mitochondria, but in an earlier complex, complex II (Avenot and Michailides, 2010; Semar et al., 2011; Stammler et al., 2007; Stammler et al., 2008; Stammler and Speakman, 2006). As with strobilurin, it was observed that boscalid also temporarily decreases plant respiration, which results in an increase in the net assimilation rate of CO<sub>2</sub> and an increase in the activity of the nitrate reductase enzyme, probably due to the acidification of the cytosol caused by the increase in the concentration of protons (H<sup>+</sup>).

In addition to the improvement of nitrogen metabolism, strobilurins also act on the hormonal balance of plants, as they reduce the synthesis of ethylene and increase the synthesis and decrease the degradation of

cytokinins. As a result, these plants present a reduction of chlorophyll degradation, causing what is called a “green effect”, delaying the yellowing of the leaves caused by the degradation of chlorophyll (Grossmann et al., 1999; Grossmann and Retzlaff, 1997; Ruske et al., 2003; Sarwat et al., 2013; Ypema and Gold, 1999). The reduction of chlorophyll degradation, combined with better nitrogen utilization, slows plant senescence and contributes to the increase in the net assimilation rate of CO<sub>2</sub>, since chlorophyll is an essential component for photosynthesis, and it absorbs energy and directs it to the photosystems (Hörtensteiner and Krätler, 2011). Increased photosynthetic activity in the leaves has been noted as the main factor for the increase of the productivity of the plants treated with strobilurins, since the delay of the senescence, even for a few days, tends to increase production (Bertelsen et al., 2001). The delay in senescence can be observed due to lower lipid peroxidation in relation to the control plants found at the end of the evaluations, indicating lower oxidative stress.

## 5. Conclusion

Based on the results obtained, it was concluded that the fungicide treatments presented different responses between grafted and ungrafted plants. The physiological benefits were more evident in the grafted plants treated with pyraclostrobin or boscalid alone, and these benefits manifested in terms of fruit production and increased the activity of the antioxidative system, thereby reducing stress. The higher productivity probably occurred due to the better physiological performance of these plants, mainly at the beginning of development, presenting greater activity of the enzyme nitrate reductase, in addition to the higher  $A_{net}$  and  $A_{net}/C_i$ .

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