

Leaves from two Solanaceae species with different leaf morphological traits respond differently to imidacloprid foliar spray*

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Abstract

Imidacloprid (IMI) is an insecticide neonicotinoid widely used for controlling crop pests and usually applied by foliar spray. The insecticide causes changes in structural and functional aspects in different plant species. However, the foliar absorption rate, which is controlled mainly by micromorphological characteristics of the leaf surface, can be related to the response intensity. This study aimed to investigate the anatomical, physiological and biochemical responses to IMI foliar application in two Solanaceae species whose leaves have distinctive micromorphological features. *Capsicum annuum* has glabrous leaves, whereas *Solanum aethiopicum* has hairy leaves. IMI was applied using leaf pulverization. Leaf absorption and wettability tests were performed. Analyses of gas exchange, chlorophyll *a* fluorescence, peroxidase and superoxide dismutase enzyme activity, and lipid peroxidation were performed. Morphometric analyses of leaf blades were performed under light microscopy. 3,3-diaminobenzidina (DAB) was used for *in situ* detection of reactive oxygen species (ROS). In both species, IMI-treated leaves showed higher mesophyll thickness and more intense ROS staining than those in untreated leaves. Imidacloprid-treated *C. annuum* leaves showed more severe alterations in physiological and biochemical aspects than those in *S. aethiopicum* leaves. This was most evident in terms of CO₂ assimilation rate, stomatal conductance, transpiration rate, and instant carboxylation efficiency. In addition, IMI-treated *C. annuum* leaves presented anatomical alterations including pronounced sinuosity on palisade parenchyma cell shape and increased intercellular spaces in spongy parenchyma. The most pronounced functional and structural alterations in IMI-treated *C. annuum* leaves was associated with their leaves micromorphological features, which increased wettability and probably IMI absorption.

1. Introduction

Insecticide application by foliar spraying is a mechanism widely used in crops in different parts of the world to improve plant productivity by preventing pest attacks. The foliar spraying of insecticides allows chemicals to enter a plant through its leaves (Fernández et al. 2021), thereby inducing changes in plant growth, physiology, and structure (Tiyagi et al. 2004; Tuffi Santos et al. 2009; Shadid et al. 2017; Wagner 1939; Petit et al. 2012). However, this method also affects non-target plants (Hatamleh et al. 2022).

Imidacloprid (IMI) belongs to a class of neonicotinoids and is commonly used to control chewing and sucking insects (Shobharani 2019). It is applied via foliar spraying, allowing for systemic absorption and translocation throughout the plant tissues (Kagabu 2003). At high doses, neonicotinoids can delay biological, physiological, and enzymatic activities, in addition to causing oxidative stress, which can lead to autolysis (Çavuşoğlu et al. 2012; Sharma et al. 2017; Shahid et al. 2021). Neonicotinoids promote changes in density and morphology of stomata, morphological changes in developing roots, cell disruption in the leaf mesophyll, and vacuolar fragmentation in root cells, in addition to affecting pollinating insect communities (Nauen et al. 2001; Schmuck et al. 2001; Çavuşoğlu et al. 2012; Whitehorn et al. 2012; Araujo 2015; Coskun et al. 2015; Zhang et al. 2020; Shahid et al. 2021; Zhang et al. 2022). Furthermore, physiological changes, including increased lipid peroxidation in leaves and increased

activity of the superoxide dismutase (SOD) enzyme in seeds, have been reported in plants treated with neonicotinoids (Dar et al. 2015; Zhang et al. 2020).

Considering that leaf permeability is related to the constitutive features of leaves (Guzmán-Delgado et al. 2018), micromorphological aspects of the leaf surface could determine the degree of leaf wettability and the consequent liquid absorption rate (Barthlott et al. 2017; Fernández et al. 2021). Therefore, differences in leaf micromorphological characteristics may result in differences in the intensity of structural and physiological responses to insecticides.

Solanaceae is an economically important angiosperm family as plants in this family are primarily used for food consumption worldwide. It comprises many species, such as tomato, potato, bell pepper, eggplant, and scarlet eggplant. Moreover, several species of the family are used to produce pharmaceuticals and drugs, as they are important alkaloid producers (Chowánski et al. 2016). Despite the widespread use of insecticides in Solanaceae crops, little is known about the effects of neonicotinoids on the structural and functional characteristics of plants from this family. Especially the effects in related plants bearing leaves with different micromorphological features.

In this study we aimed to investigate the anatomical, physiological, and biochemical responses of Solanaceae species bearing leaves with different micromorphological features to foliar application of imidacloprid. For this, we used two Solanaceae species as models. The first is *Capsicum annuum* L., popularly known as bell pepper; it is a bushy plant with simple and glabrous leaves (Aguilar-Rincón et al. 2010) that exhibit an irregular surface with depression areas (personal observation). The second is *Solanum aethiopicum* L. var. Gilo, popularly known as scarlet eggplant; it is a bushy plant with simple and hairy leaves (Lester and Daunay 2003; Bello et al. 2017; Watts and Kariyat 2021) that exhibit a regular surface. We hypothesized that species with glabrous leaves with an irregular surface would have different wettability rates and, therefore, different structural and functional responses to IMI application compared with hairy leafed species.

2. Material and methods

2.1 Greenhouse experiment

Seeds of TopSeed Garden[®], from both species were spread on acrylic trays that contained vermiculite. Thirty days after germination, the plants were transplanted into 800-mL pots filled with Carolina[®] substrate, containing *Sphagnum* peat, expanded perlite, expanded vermiculite, and toasted rice husk. The pots were kept in a Pad & Fan greenhouse (Van der Hoeven Estufas Agrícolas Ltda.) in the Institute of Biosciences of Botucatu of the São Paulo State University (UNESP) and were irrigated every two days until water percolation. After 15 days of acclimatization, when the aerial axes of the plants were approximately 35.0 cm high, Evidence[®] 700WG by Bayer[®], 1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine (IMI), was applied.

We conducted randomized blocks with two treatments: a) foliar spraying of water + Haiten® adjuvant (a solution of polyoxymethylene, phenol, and ether) as the control, and b) foliar spraying of pesticide + Haiten® adjuvant. Ten specimens from each species ($n = 10$) were subjected to each treatment. The total surface area of the pots was calculated in hectares (ha) to obtain the ratio. A manual costal sprayer (CO_2) with a 101 SP 04 nozzle was used to apply the pesticide, with a dosage of 200 mL/ha, including 0.05% of Haiten® non-ionic adjuvant (Arysta Lifescience do Brasil, Indústria Química e Agropecuária Ltda.). The pesticide was applied in three rounds, with a seven-day interval between each application, in accordance with the manufacturer's recommendations to control the insect *Thrips palmi* Karny.

2.2 Physiological data evaluation

On the day before the first application and every two days after treatment (DAT) (at zero, three, ten and 17 days), both gas exchange rates and chlorophyll *a* fluorescence were measured between 8:00 and 11:00 am in fully expanded leaves located at the third cauline node (Ramos et al. 2015).

Chlorophyll *a* fluorescence was measured using a fluorometer attached to the Infra-Red Gas Analyzer (IRGA, LI-6400, Li-Cor) using the saturated pulse method (Maxwell and Johnson 2000). Briefly, the leaves were covered with aluminum foil to be kept in darkness for 30 min before recording the fluorescence. The minimal fluorescence (F_0) was determined under sufficiently low irradiation ($< 1 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). The maximum fluorescence (F_m) was determined after a saturation pulse of 0.8 s at $4.200 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. In light-adapted leaves, the radiation from saturation pulses to determine maximum fluorescence (F_m') was $6.000 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ for 0.8 s, and the actinic light was $200 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Photosystem II (PSII) photochemical quantum yield was measured by applying a saturation light pulse ($6 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ for 0.8 s) under ambient irradiation. Fluorescence parameters such as the maximum variable quantum yield of PSII (F_v/F_m), effective quantum yield of PSII (F_v'/F_m'), photochemical quenching (qP), non-photochemical quenching (qNP), and relative electron transport rate (ETR) were calculated according to a methodology described by Schreiber et al. (1986). For the calculation of ETR , the fraction of excitation energy distributed to PSII was considered to be 0.5 and the fraction of photosynthetically active photon flux density absorbed by the leaves were 0.84 (Demmig and Bjorkman 1987).

Measurements of gas exchange were carried out using equipment with an open system of photosynthesis with a CO_2 analyzer and water vapor by infrared radiation (Infra-Red Gas Analyzer- IRGA, Li-Cor 6400, USA). The CO_2 assimilation rate (A , $\mu\text{molCO}_2\text{m}^{-2}\text{s}^{-1}$), transpiration rate (E , $\text{mmol water vaporm}^{-2}\text{s}^{-1}$), stomatal conductance (g_s , $\text{molm}^{-2}\text{s}^{-1}$), and internal CO_2 concentration (C_i , $\mu\text{molCO}_2\text{mol}^{-1}\text{air}$) were measured in three plants per treatment. The CO_2 concentration that entered the chamber (LCF chamber; 2 cm^2 , Li-Cor) averaged $400 \mu\text{mol} \cdot \text{mol}^{-1}$, as provided by the 6400-01 CO_2 mixer (Li-Cor). The Photosynthetic Photon Flux Density (PPFD) was provided using a light emitting diode (LED) as the light source (6400-40 LCF, Li-Cor, 90% red and 10% blue spectrum) and set to deliver $1,000 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ in the chamber, based on the previously performed light curve. The vapor pressure deficit (VPD) inside the chamber was $2.08 \pm 0.18 \text{ kPa}$, which means that the relative humidity in the chamber (sample) was

65.1 ± 2.3%. Water use efficiency (WUE , $\mu\text{mol CO}_2 / \text{mmol}^{-1} \text{H}_2\text{O m}^{-2} \text{s}^{-1}$) was calculated using the relationship between CO_2 assimilation rate and transpiration. Instant carboxylation efficiency (A/C_i) was determined by the relationship between the CO_2 assimilation rate and CO_2 internal concentration in the leaf.

2.3 Enzyme activity analysis

For biochemical analyses, fully expanded and non-senescent leaves were collected from four specimens of each species per treatment, between 8:00 and 11:00 am. The samples were instantly frozen in liquid nitrogen and kept at -80°C in an ultrafreezer. In the laboratory, leaves were macerated with liquid nitrogen. Of the pulverized material, 300 mg was weighed for enzymatic extraction and 200 mg was weighed for lipid peroxidation analysis. The entire process was conducted using pre-cooled tubes and tweezers to prevent sample thawing.

The enzymes were extracted according to the method proposed by Kar and Mishra (1976), by adding 5.0 mL of a solution containing 50.0 mM phosphate buffer (pH 6.7), and 1% (w/v) polyvinylpolypyrrolidone. The samples were homogenized by centrifugation at 10,000 rpm for 10 mins at 4°C , and the supernatant was collected for analysis. The soluble proteins of each enzyme extract were quantified according to a methodology proposed by Bradford (1976) to determine the specific activity of the antioxidant enzymes.

Superoxide dismutase SOD activity was determined based on the ability of the enzyme to inhibit nitroblue tetrazolium (NBT) photoreduction, according to the methodology proposed by Giannopolitis and Ries (1977), with modifications by Dousseau et al., (2016). Absorbance was read at 560 nm using a BiotekEpoch™ Microplate ELISA Spectrophotometer. A unit of SOD activity was defined as the amount of enzyme required to inhibit NBT photoreduction by 50% under the assay conditions, expressed as U mg protein⁻¹.

Peroxidase (POD) enzyme activity was determined according to the methodology proposed by Teisseire and Guy (2000). Adaptations and readings were performed at 430 nm using a BiotekEpoch™ Microplate ELISA Spectrophotometer. The molar extinction coefficient was used to calculate POD activity ($2.5 \text{ mmol} \cdot \text{l}^{-1} \cdot \text{cm}^{-1}$), expressed as $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg} \cdot \text{protein}^{-1}$.

2.4 Lipid peroxidation index measurement

Lipid peroxidation index (MDA) was measured according to the technique proposed by Rama Devi and Prasad (1998). Macerated leaf samples (200 mg) were homogenized in 5.0 mL extraction buffer containing 0.25% thiobarbituric acid and 10% trichloroacetic acid, and then boiled at 95°C for 30 min. The reaction mixture was cooled on ice and centrifuged at 10,000 rpm for 5 min. Absorbance was measured at 560 nm and 600 nm, and the results were expressed as μmol of MDA per gram of fresh mass.

2.5 Light microscopy

For anatomical investigation, fully expanded leaves formed under the experimental conditions were collected and fixed in a solution of 37% formaldehyde, glacial acetic acid, and 50% ethanol (5:5:90) for 24 h (Johansen 1940). Samples from the middle region of the leaf blade were dehydrated in an ethylic series and embedded in Leica methacrylate resin (Gerrits 1991). Next, transverse sections (5.0 μm) were obtained using a Leica RM2145 semi-automatic rotary microtome and stained with 0.05% Toluidine Blue pH 4.7 (O'Brien et al. 1964). Permanent slides were mounted using synthetic resin as mounting medium. The slides were analyzed using a Leica DMR photomicroscope, and the results were documented using a Leica DFC425 digital camera.

Morphometric analyses were conducted under the light microscope using LAS software, version 3.8. The following parameters were measured: thickness of the epidermis on the adaxial and abaxial sides of the leaf blade; thickness of the external periclinal wall of epidermal cells on the adaxial surface of the leaf blade; thickness of the mesophyll; thickness of the palisade and spongy parenchyma; area occupied by intercellular spaces in the spongy parenchyma; distance between third-order veins; and density of calcium idioblasts.

2.6 Scanning electron microscopy (SEM)

For the analysis of the leaf blade surface, samples from the middle region of the blade of fully expanded leaves formed under the experimental conditions were fixed in Karnovsky solution (4% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer pH 7.2; 0.2 M phosphate buffer pH 7.2) for 24 h (Karnovsky 1965). Subsequently, they were fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.3) for 1 h and dehydrated in an ethyl series. The samples were critical point dried (CPD) to 020 (Balzer Union) using liquid CO_2 , glued onto aluminum supports, and metallized with gold. Analyses were performed using a FeiQuanta scanning electron microscope at 20 kV.

2.7 Histochemical tests

Freshly collected leaf blades were sectioned using razor blades and treated with 10% hydrochloric acid for identification of oxalate of calcium in the crystals inside idioblasts. For histological localization of reactive oxygen species (ROS), leaf blades were kept in a 0.5% solution of 3,3-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffer (pH 7.4) for 60 min in the dark. For the control test, samples were kept in an ascorbic acid solution for the same period. Next, the samples were sectioned using razor blades and, analyzed using a Leica DMR photomicroscope coupled to a Leica DFC425 digital camera.

2.8 Leaf absorption

A drop of 200 μL of 1% aqueous safranin was deposited on the adaxial surface of the blade of fully expanded leaves located at the third node below the apex stem, and the solution remained on the leaf surface until it was completely dried. Next, the leaves, still attached to the plant, were washed with 90% ethanol until the dye was completely removed from the leaf surface, and then washed in running water. After detachment from the plants, the treated region was transversally sectioned with razor blades

(Milanez and Machado 2008 - adapted). The sections were mounted between slides and coverslips using water and analyzed under a Leica TCSSP5 laser scanning confocal microscope, with excitation at 514 nm and detection between 559 and 605 nm.

2.9 Wettability analysis

Different wettability tests were conducted using deionized water (pH 6.5) at 19°C: a) deionized water; b) deionized water + adjuvant; c) deionized water + IMI + adjuvant. Five leaves of each species were used. Each newly collected leaf was attached to a flat Styrofoam plate using an adhesive tape. A 5.0 µL drop of liquid was deposited on the adaxial surface of each leaf using a micropipette (Matos and Rosado, 2016). A photographic camera was used to record the results, and the contact angle between the drop of liquid and the leaf surface was calculated using ImageJ software, version 1.53. The leaves were classified according to the wettability criterion presented by Fernández et al. (2021): wettable leaves have contact angles between the droplet and the leaf surface smaller than 90°; in non-wettable leaves, the contact angle between the drop and the leaf surface is greater than or equal to 90°; leaves where the contact angle between the droplet and the leaf surface is greater than or equal to 130° are considered highly non-wettable.

2.10 Statistical analyses

For each treatment, data normality was initially checked using the Anderson-Darling test, with subsequent verification of homoscedasticity (homogeneity of variances) using Hartley's test. The data were then subjected to an analysis of variance at a 5% significance level of error probability. When significant, means were compared using Tukey's test at 5% error probability. For quantitative variables, a regression analysis was used, adopting a 5% error probability. R statistical software (version 4.1.2) and the graphical environment of RStudio software (version 2021.09.2 Build 382) were used. Graphs were drawn using the SigmaPlot software (version 14.0).

3. Results

3.1 Chlorophyll *a* fluorescence and physiological analyses

3.1.1 *Capsicum annuum*

In the control-treated *C. annuum* plants (F = not significant 'ns') the *qP* decreased at three DAT, increased at ten DAT, and decreased at 17 DAT, whereas in IMI-treated plants, (F = 11.8) the *qP* increased at three and ten DAT and a decreased at 17 DAT (Fig. 1A). The *qNP* decreased at three DAT and increased at ten DAT in both the control (F = ns) and IMI-treated (F = ns) plants; however, the *qNP* decreased at 17 DAT in control plants and slightly increased in IMI-treated plants (Fig. 1b). The *Fv'/Fm'* increased at three DAT and decreased at ten DAT in both treatments (F = ns); however, control plants continued to show decreasing *Fv'/Fm'* at 17 DAT, whereas IMI-treated plants showed stabilization of *Fv'/Fm'* at 17 DAT

(Fig. 1C). The Fv/Fm decreased at three and ten DAT and increased at 17 DAT in plants from both treatment groups ($F = 8.23$ control; $F = ns$ IMI) (Fig. 1D).

The A increased at three DAT and decreased exponentially at ten and 17 DAT in control plants ($F = 18.2$); in IMI-treated plants, ($F = 7.2$) the A decreased linearly throughout the experiment (Fig. 2A). Regarding the stomatal conductance, plants from both the control ($F = 62.3$) and IMI treatment ($F = ns$) groups showed increased g_s at three DAT and decreased g_s at ten DAT; however, control plants continued to show reduction of g_s at 17 DAT, whereas IMI-treated plants showed g_s stabilization at 17 DAT (Fig. 2B). The Ci increased throughout the treatment in control plants ($F = 5.98$); in IMI-treated plants ($F = ns$), the Ci increased at three DAT, decreased at ten DAT, and increased at 17 DAT (Fig. 2C). The E increased in control plants ($F = 15.52$) at three DAT and decreased exponentially at ten and 17 DAT; in IMI-treated plants ($F = 17.2$), the E increased at three DAT, decreased at ten DAT, and increased slightly at 17 DAT (Fig. 2D).

The ETR increased at three DAT and decreased at ten DAT in plants from both control and IMI treatment groups; however, the ETR continued to decrease in control plants ($F = 9.3$) and stabilized in the IMI-treated plants ($F = ns$) at 17 DAT (Fig. 3A). The A/Ci increased at three DAT and decreased exponentially at ten and 17 DAT in the control plants ($F = 40.1$); in IMI-treated plants ($F = 24.0$), the A/Ci decreased throughout the experiment (Fig. 3B). The WUE decreased linearly in control plants ($F = 16.97$) throughout the experiment; in IMI-treated plants ($F = ns$), the WUE increased at three DAT, decreased at ten DAT, and stabilized at 17 DAT (Fig. 3C).

3.1.2 *Solanumaethiopicum*

In *S. aethiopicum* plants from the control treatment ($F = ns$), the qP remained stabilized throughout the experiment; in IMI-treated plants ($F = 12.3$), the qP increased at three and ten DAT and decreased at 17 DAT (Fig. 1A). The qNP increased throughout the experiment in plants from both control ($F = 8.6$) and IMI treatment groups ($F = 9.5$ IMI) (Fig. 1B). The Fv'/Fm' increased linearly throughout the experiment in plants from the control group ($F = 7.8$); IMI-treated plants ($F = ns$) showed stabilization of Fv'/Fm' at three and ten DAT and an increase of Fv'/Fm' at 17 DAT (Fig. 1C). The Fv/Fm decreased at three and ten DAT and increased at 17 DAT in plants from both control ($F = 7.34$) and IMI treatment groups ($F = ns$) (Fig. 1D).

Both the A ($F = 11.3$ control; $F = ns$ IMI) (Fig. 2A) and g_s ($F = 43.2$ control; $F = ns$ IMI) (Fig. 2B) increased linearly throughout the experiment in control plants; in IMI-treated plants, A and g_s increased at three DAT, decreased at ten DAT, and increased at 17 DAT. The Ci decreased at three DAT and increased at ten and 17 DAT in control plants ($F = 6.16$); in IMI-treated plants ($F = ns$), the Ci increased at three DAT, decreased at ten DAT, and increased at 17 DAT (Fig. 2C). The E increased at three and ten DAT and decreased at 17 DAT in control plants ($F = 19.4$); in IMI-treated plants ($F = ns$), the E increased at three DAT, decreased at ten DAT, and stabilized at 17 DAT (Fig. 2D).

The ETR increased linearly throughout the experiment in plants from the control treatment group ($F = 10.3$); in IMI-treated plants ($F = 12.8$), the ETR increased at three and ten DAT and decreased at 17 DAT

(Fig. 3A). The A/C_i increased at three and ten DAT and decreased at 17 DAT in control plants ($F = 12.36$); in IMI-treated plants ($F = ns$), the A/C_i increased at three DAT, decreased at ten DAT, and stabilized at 17 DAT (Fig. 3B). The WUE decreased at three DAT, stabilized at ten DAT, and increased at 17 DAT in control plants ($F = 6.32$), whereas in IMI-treated plants ($F = ns$), the WUE increased at three DAT, decreased at ten DAT, and increased at 17 DAT (Fig. 3C).

3.2 Biochemical analyses

3.2.1 *Capsicum annuum*

Lipid peroxidation increased throughout the experiment in plants from the control treatment ($F = 48.2$) and in IMI-treated plants ($F = 25.4$ IMI) (Fig. 4A). POD activity decreased slightly in control plants ($F = 31.17$); in IMI-treated plants ($F = 15.02$), the POD activity decreased at three DAT and decreased slightly at ten and 17 DAT (Fig. 4B). SOD activity increased at three and ten DAT and decreased at 17 DAT in control plants ($F = ns$); in IMI-treated plants ($F = 36.14$), the SOD activity increased slightly throughout the experiment (Fig. 4C).

3.2.2 *Solanum aethiopicum*

Lipid peroxidation decreased at three DAT, increased at ten DAT, and decreased at 17 DAT in control plants ($F = ns$); in IMI-treated plants ($F = 7.64$), the lipid peroxidation decreased at three DAT, decreased slightly at ten DAT, and increased slightly at 17 DAT (Fig. 4A). POD activity decreased exponentially in the control plants ($F = 33.9$); in IMI-treated plants ($F = 62.6$), POD activity also decreased throughout the experiment, albeit not intensely (Fig. 4B). SOD activity increased linearly throughout the experiment in control plants ($F = 22.3$); in IMI-treated plants ($F = 31.17$), SOD activity increased slightly during the experiment (Fig. 4C).

3.3 Comparisons between IMI-treated *Capsicum annuum* and *Solanum aethiopicum* plants

At the end of the experimental period, IMI-treated *S. aethiopicum* plants showed higher F_v'/F_m' than that of IMI-treated *C. annuum* plants (Fig. 1C). Despite a decrease over the course of the experiment, the values of F_v/F_m increased at the end of the experiment in IMI-treated plants from both species (Fig. 1D). The IMI-treated *C. annuum* plants exhibited lower A and g_s than that of IMI-treated *S. aethiopicum* plants at the end of the experimental period (Fig. 2A, B). The IMI-treated *S. aethiopicum* plants showed higher C_i (Fig. 2C) and higher E (Fig. 2D) than that of IMI-treated *C. annuum* plants.

3.4 Leaf morphology

3.4.1 Leaf surface

In the frontal view the leaf surface of *S. aethiopicum* was regular in control (Fig. 5A) and IMI-treated (Fig. 5B) plants. The common epidermal cells on the adaxial and abaxial surface of the blade showed a

slightly sinuous contour and were covered by a smooth cuticle in both control (Fig. 5A) and IMI-treated plants (Fig. 5B). Stomata and non-glandular and glandular trichomes were observed on both sides of the blade in both control (Fig. 5A) and IMI-treated plants (Fig. 5B). In the cross sections, the epidermis was uniseriate, with cells ranging from rounded to slightly tangentially elongated on the adaxial surface of the leaf blade, whereas on the abaxial surface of the leaf blade, the epidermal cells were less voluminous than those on the adaxial surface and had a rounded to rectangular shape (Fig. 5C, D) in both treatments.

The surface of the leaf blade surface of *C. annuum* in the frontal view showed an irregular relief, especially on the abaxial side. Groups of epidermal cells were arranged at different levels, both in the control (Fig. 5E) and IMI-treated plants (Fig. 5F), forming slightly more salient and deeper zones on the leaf surface. The cuticle on the leaf surface was thin and smooth in both the control (Fig. 5E) and IMI-treated plants (Fig. 5F); slight cuticle striation was observed in cells adjacent to the stomata (Fig. 5E, F). The leaves were amphistomatic and the epidermis was uniseriate (Fig. 5G, H). On the adaxial surface of the leaf blade, the common epidermal cells were voluminous, with a rounded to slightly tangentially elongated shape in plants of both treatments (Fig. 5G, H).

The thickness of the external periclinal wall on the adaxial blade surface (Fig. 6A) and the height of epidermal cells on the adaxial (Fig. 6B) and abaxial blade surfaces (Fig. 6C) showed no variation between specimens from the control and IMI-treated groups in either species.

3.4.2 Mesophyll

Both species presented dorsiventral mesophylls, in both the control (Fig. 5C, G) and IMI-treated plants (Fig. 5D, H). A layer of palisade parenchyma was observed underlying the epidermis on the adaxial blade surface in both the species (Fig. 5C, G). However, the palisade parenchyma cells in IMI-treated *S. aethiopicum* plants had periclinal division, resulting in a locally biseriate structure (Fig. 5D). Control plants of *C. annuum* presented palisade parenchymal cells with a slightly sinuous outline (Fig. 5G). IMI-treated plants presented palisade parenchymal cells with evident sinuosity (Fig. 5H).

The spongy parenchyma presented 4–5 layers of cells in *S. aethiopicum* plants (Fig. 5C, D), and 3–4 layers in *C. annuum* plants (Fig. 5G, H), both in the control and IMI treatment groups. The intercellular spaces in the spongy parenchyma of the IMI-treated *S. aethiopicum* plants were smaller than those in the control plants (Fig. 7A). In contrast, in IMI-treated *C. annuum* plants, a larger mesophyll area occupied by intercellular spaces than that in control plants was observed (Fig. 7A).

IMI-treated plants of both species presented thicker spongy and palisade parenchyma (Fig. 7B, C) and thicker mesophyll (Fig. 7D) than those in the control plants.

The control and IMI-treated plants from both species presented idioblasts with sandy crystals in spongy parenchyma (Fig. 5C, D, G, H). The results of the test with a 10% hydrochloric acid solution indicated that these crystals comprised calcium oxalate. The IMI-treated plants of *S. aethiopicum* and *C. annuum* had a higher density of crystal idioblasts than did the control plants (Fig. 7E).

3.4.3 Vascular system

Collateral vascular bundles occurred immersed in the leaf mesophyll of both species in both treatment groups (Fig. 5C, D, G, H). In both species, the distance between third-order veins was smaller in IMI-treated leaves than in control plant leaves (Fig. 7F).

3.5 Histochemical detection of ROS

ROS were histochemically detected in the leaves of both species in both treatment groups (Fig. 8A–H). All leaf blade tissues were labeled, as observed in common epidermal cells, palisade parenchyma, spongy parenchyma, phloem, and xylem (Fig. 8A–H). In *S. aethiopicum*, ROS were also detected in the non-glandular (Fig. 8A–D) and glandular trichomes (Fig. 8B, D). In both species, IMI-treated plants (Fig. 8C, D, G, H) showed more intense staining for ROS than the control plants (Fig. 8A, B, E, F).

3.6 Leaf absorption

The foliar absorption test showed safranin uptake in the epidermis, mesophyll parenchyma cells, and vascular tissues of both species (Fig. 9A, B). In *S. aethiopicum*, absorption was also observed in the non-glandular trichomes (Fig. 9A).

3.7 Wettability

The wettability tests showed varying results based on the mean deposition angle of the water droplet in relation to the leaf surface. The deionized water test showed angles of 102° in *S. aethiopicum* and 86° in *C. annuum*; hence, they were considered to be non-wettable and wettable leaves, respectively. The deionized water + adjuvant test showed angles of 34° in *S. aethiopicum* and 40° in *C. annuum*; therefore, both leaves were classified as wettable. Finally, the deionized water + IMI + adjuvant test presented angles of 42° in *S. aethiopicum* and 48° in *C. annuum*, thereby giving the wettable trait to the leaves of both species.

4. Discussion

In general, our results showed that leaves of *C. annuum* and *S. aethiopicum* treated with IMI presented anatomical, physiological, and biochemical changes compared with the control plants. Moreover, more severe changes than those in *S. aethiopicum* were registered in *C. annuum*.

The physiological analyses showed reduced gas exchange rates in *C. annuum* throughout the treatment period, both in the control and IMI-treated plants. However, *Ci* increased even in plants with low stomatal g_s , which may indicate that the carboxylase function of the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) would vary, leading to CO₂ accumulation. This suggests that the carboxylase activity of the Rubisco enzyme was changed, probably due to resistance to CO₂ passage to the sites of action of this enzyme (Busch 2020). Furthermore, IMI-treated *C. annuum* and *S. aethiopicum* plants reduced *qP*. This may indicate a risk of damage to the photosynthetic apparatus owing to a

reduced ability to protect it, as the captured energy cannot be satisfactorily dissipated (Silva et al. 2016). This could lead to increased ROS levels, causing oxidative stress in the studied plants. Plants have evolved a complex antioxidant system that acts to repair the damage caused by ROS (Dar et al. 2015). Histochemical DAB tests for histological localization of ROS produced intense labeling in plants treated with IMI in both species. High ROS concentrations can cause molecular damage to lipids, proteins, and DNA (Juan et al. 2021). This damage changes membrane properties, such as fluidity, ion transport, loss of enzymatic activities, and inhibition of protein synthesis, and may even lead to cell death (Sharma et al. 2012).

The ability of plants to mitigate oxidative stress partially depends on the induction of SOD activity and, subsequently, on the regulation of other derived antioxidant enzymes (Alscher et al. 2002). The SOD enzyme is the first line of plant-defense against ROS (Liang et al. 2003). *S. aethiopicum* and *C. annuum* plants treated with IMI had lower SOD enzyme activity than that in control groups, unlike what has been reported for other plant species (Dar et al. 2015; Homayoonzadeh et al. 2020). This may explain the increased ROS accumulation detected by the DAB test. However, even if lower than that in control plants, the IMI-treated specimens of both species showed increased SOD activity throughout the experiment. Thus, SOD activity in *S. aethiopicum* plants maintained relatively stable lipid peroxidation rates, with IMI having an elicitor role in generating defense response (Alscher et al. 2002).

POD enzyme activity decreased in both species throughout the experiment, in both control and IMI-treated plants. However, from the beginning of the treatment, *C. annuum* showed lower activity than *S. aethiopicum* of this enzyme. Decreased antioxidant enzyme activity can lead to increased lipid peroxidation and, damage to cell membranes (Hernandez et al. 2000). Even with decreased POD activity, *S. aethiopicum* did not exhibit increased lipid peroxidation rates. In contrast, *C. annuum* plants showed increased lipid peroxidation rates, which may have led to the pronounced anatomical changes recorded in this study.

When metabolized by plants, IMI generate two main metabolites, 6-chloropyridinyl-3-carboxylic and 6-chloro-2-hydroxypyridinyl-3-carboxylic acids, both of which are salicylic acid analogs (Ford et al. 2010). These acids induce acquired systemic resistance (Szczepaniec et al. 2013). The application of salicylic acid can increase the activity of antioxidant enzymes, such as SOD and POD, reduce lipid peroxidation, protect the photosynthetic apparatus, improve g_s , and increase A in tomato plants (Aires et al. 2022). Thus, *S. aethiopicum* plants under the effect of IMI showed responses similar to those of plants treated with salicylic acid, which may indicate a possible antioxidant action of this insecticide in this species. However, *C. annuum* plants treated with IMI, despite the increased SOD concentration, showed increased peroxidation rates, suggesting that the increased concentration of this enzyme was insufficient to stop oxidative processes.

The leaves of *C. annuum* IMI-treated plants showed mesophyll cells with a very sinuous contour, which may have resulted in the largest area of intercellular spaces registered in the mesophyll of these leaves. As already verified for other plant species, plants treated with IMI may show morphological changes in

mesophyll cells (Zhang et al. 2020). Considering that IMI can change the formation of structural components in animal cells (Gao et al. 2016), conformational changes in *C. annuum* chlorophyll parenchyma cells may be related to configuration problems in cytoskeletal elements, which are essential for the formation and maintenance of the cell wall structure (Chebli et al. 2021). However, no studies have investigated the effects of IMI on the cytoskeleton of plant cells.

IMI-treated plants of both species showed reduced distances between the veins immersed in the leaf mesophyll. Plants exposed to toxic substances, such as herbicides, have a reduced distance between vascular bundles, which may be associated with increased translocation of these substances within the plant body (Marques et al. 2012). This may be an attempt to eliminate this compound by promoting its distribution to sites of substance degradation (Rocha 2001).

Plants from both species treated with IMI showed increased mesophyll thickness compared with that in the control plants, and in *S. aethiopicum*, the palisade parenchyma became locally biseriate. The increased thickness of the palisade and spongy parenchyma of plants under stress conditions may favor the maintenance of photosynthetic rates by increasing assimilation sites and improving CO₂ diffusion in the leaves (Ennajeh et al. 2010). Changes in mesophyll thickness significantly improve CO₂ capture and diffusion in conditions of reduced g_s (Violet-Chabrand et al. 2017; Toscano et al. 2018).

Plants treated with IMI may show increased calcium and zinc concentrations in the leaves due to the activation of different metabolic pathways (Zhang et al. 2020). In the present study, calcium concentration in the tissues was not measured; however, considering studies that reported increased calcium concentrations in plants after IMI application (Zhang et al. 2020), it is likely that this may have occurred in *C. annuum* and *S. aethiopicum*. An abundance of idioblasts containing calcium oxalate crystals in the mesophyll of IMI-treated plants suggests that the concentrations of calcium in *C. annuum* and *S. aethiopicum* leaves increased. Some studies have shown that excessive calcium is toxic to plant cells and tissues, and that this excess tends to be immobilized as crystals in different tissues (Zindler-Frank et al. 2001; Mazen et al. 2003; Franceschi and Nakata 2005; Smith et al. 2009).

In summary, our results demonstrate that IMI-treated *C. annuum* plants present more severe physiological and biochemical changes than those in IMI-treated *S. aethiopicum*. This is especially evident in terms of A , g_s , E , and A/C_i as well as structural changes, such as increased contour sinuosity in palisade parenchyma cells and increased area occupied by intercellular spaces in the spongy parenchyma. The most pronounced functional and structural changes in *C. annuum* in response to IMI application may be associated with its significant wettability and consequent IMI absorption. These results corroborate with those of Weryszko-Chmielewska and Michalojc (2009), who reported that the outer periclinal wall and thin cuticle of the epidermal cells of *C. annuum* leaves do not seem to be barriers to the penetration of substances applied to the leaf surface.

Our results showed that *S. aethiopicum* and *C. annuum* leaves can absorb substances through their surfaces, and that in the situations where an adjuvant is used, both are wettable. However, there were

differences in the wettability between the species in the absence of adjuvant. In the wettability test exclusively using water, *C. annuum* leaves were considered wettable, whereas *S. aethiopicum* leaves presented as non-wettable. The structural characteristics of the leaf surface can interfere with the wettability of the leaves and affect the absorption or repellency of liquids (Alves 2017; Schreel et al. 2020). Moreover, water attraction needs to be greater than the surface tension for good wettability (Lo and Hopkinson 1995; Moita Neto 2006). The absence of trichomes associated with the cuticle striations and surfaces with irregular relief are liable for the accumulation of liquid substances on *C. annuum* leaves, thereby favoring their wettability. In contrast, *S. aethiopicum* leaves presented regular and smooth surfaces, in addition to trichomes. Ongoing studies (Nicolai and Rodrigues *in prep*) using histochemical tests have shown that the body and arms of non-glandular trichomes in *S. aethiopicum* leaves are formed by cells with lignified walls, which could interfere with the wettability of this species and hinder the absorption of hydrophilic substances, such as IMI, through the leaf surface. However, it should be noted that, in the present study, adjuvant was used during IMI applications. Adjuvant in association with chemical defensive could function as buffers, dispersants, emulsifiers, wettable, adhesives, and spreads (Oliveira 2011). Surfactant adjuvants, as used in this study, facilitate the water drop spreading and decreasing drop contact angle trough the leaf surface, by making the droplet lose its spherical shape (Singh and Mack 1993). Thus, even *S. aethiopicum* leaves were shown to be non-wettable in the test using water; however, they were wettable in the wettability test with the adjuvant and therefore, able to absorb the insecticide through the leaf surface.

Therefore, although the presence or absence of trichomes appears to be one of the factors interfering with leaf wettability, other morphological aspects, such as the constitution of epidermal appendages and relief of the leaf surface, seem to effectively interfere with the absorption conditions on the leaf surface. Thus, studies involving numerous phylogenetically related species are required to understand the role of trichomes in the absorption of substances on the leaf surface.

5. Conclusions

In both species, IMI-treated leaves showed higher mesophyll thickness and more intense ROS staining than those in untreated leaves. IMI-treated *C. annuum* leaves showed more severe alterations in physiological and biochemical aspects than those in *S. aethiopicum* leaves. This was most evident in terms of CO₂ assimilation rate, stomatal conductance, transpiration rate, and instant carboxylation efficiency. In addition, IMI-treated *C. annuum* leaves presented anatomical alterations including pronounced sinuosity on palisade parenchyma cell shape and increased intercellular spaces in spongy parenchyma. The most pronounced functional and structural alterations in IMI-treated *C. annuum* leaves was associated with the micromorphological features of their leaves, which increased wettability and probably the IMI absorption.

Abbreviations

IMD: imidacloprid; SOD: superoxide dismutase; DAT: days after treatment; PSII: Photosystem II; ETR: electron transport rate; WUE: water use efficiency; NBT: nitroblue tetrazolium; POD: peroxidase; MDA: lipid peroxidation index; SEM: Scanning electron microscopy; ROS: reactive oxygen species; DAB: 3,3-diaminobenzidine; ns: not significant; *F_m*: maximum fluorescence; *qP*: photochemical quenching; *qNP*: non-photochemical quenching; PSII: Photosystem II; *F_v/F_m*: maximum variable quantum yield of PSII; *F_v'/F_m'*: effective quantum yield of PSII; *g_s*: stomatal conductance; *A*: CO₂ assimilation rate; *C_i*: internal CO₂ concentration; *E*: transpiration rate; *A/C_i*: instantaneous carboxylation efficiency; LCF: CO₂ concentration in chamber; Rubisco: ribulose 1,5-bisphosphate carboxylase/oxygenase

Declarations

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Author contributions

J Nicolai and TM Rodrigues designed the study and analyzed and interpreted the anatomical data. J Nicolai, ES Aires, ICS Marques, and FGBF Freitas Júnior performed the biochemical and physiological analysis. J Nicolai, EO Ono and ES Aires analyzed and interpreted the biochemical and physiological data. FF Putti performed the statistical analysis. J Nicolai wrote the manuscript with contributions from all authors.

Declaration of competing interest

The authors have no competing interests to declare.

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Figures

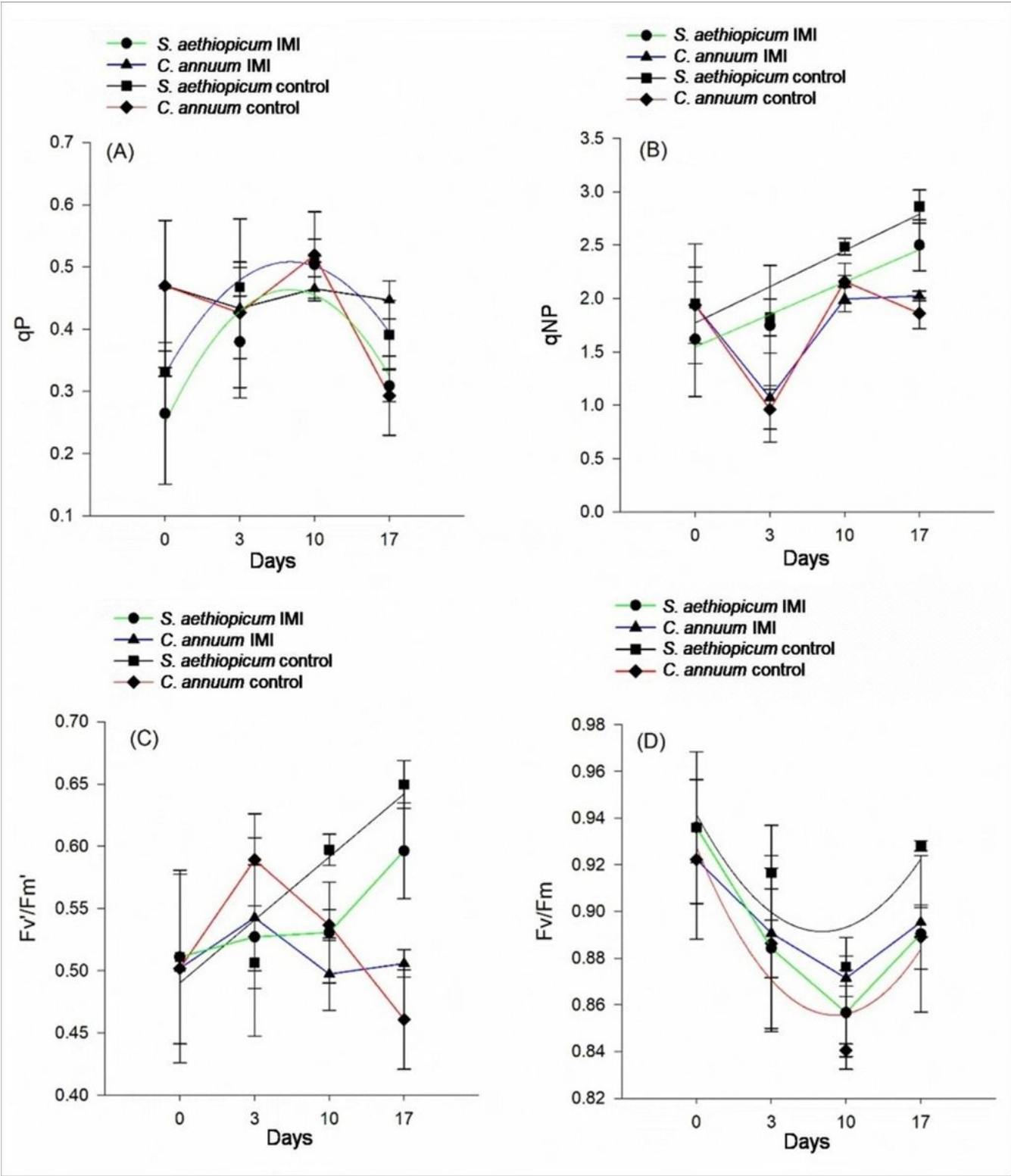


Figure 1

Graphical representation of the effect of imidacloprid (IMI) on chlorophyll *a* fluorescence in *Solanum aethiopicum* and *Capsicum annuum* plants throughout the experimental period. *qP*: photochemical quenching; *qNP*: non-photochemical quenching; *Fv'/Fm'*: effective quantum yield of PSII; *Fv/Fm*: maximum variable quantum yield of PSII.

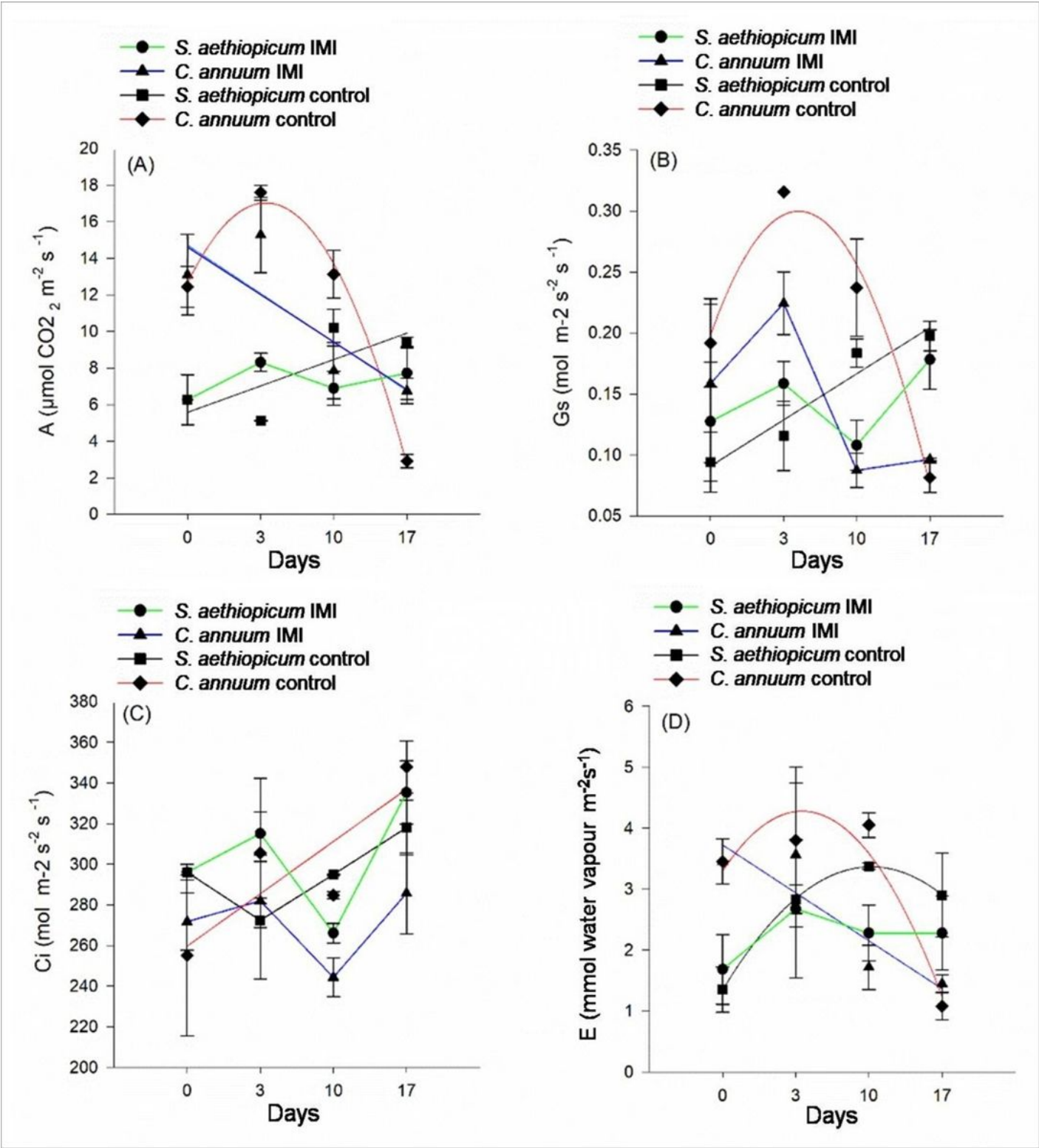


Figure 2

Graphical representation of the effect of imidacloprid (IMI) on gas exchange indices of *Solanum aethiopicum* and *Capsicum annuum* plants throughout the experimental period. A: CO₂ assimilation rate; *g_s*: stomatal conductance; *C_i*: internal CO₂ concentration; *E*: transpiration rate.

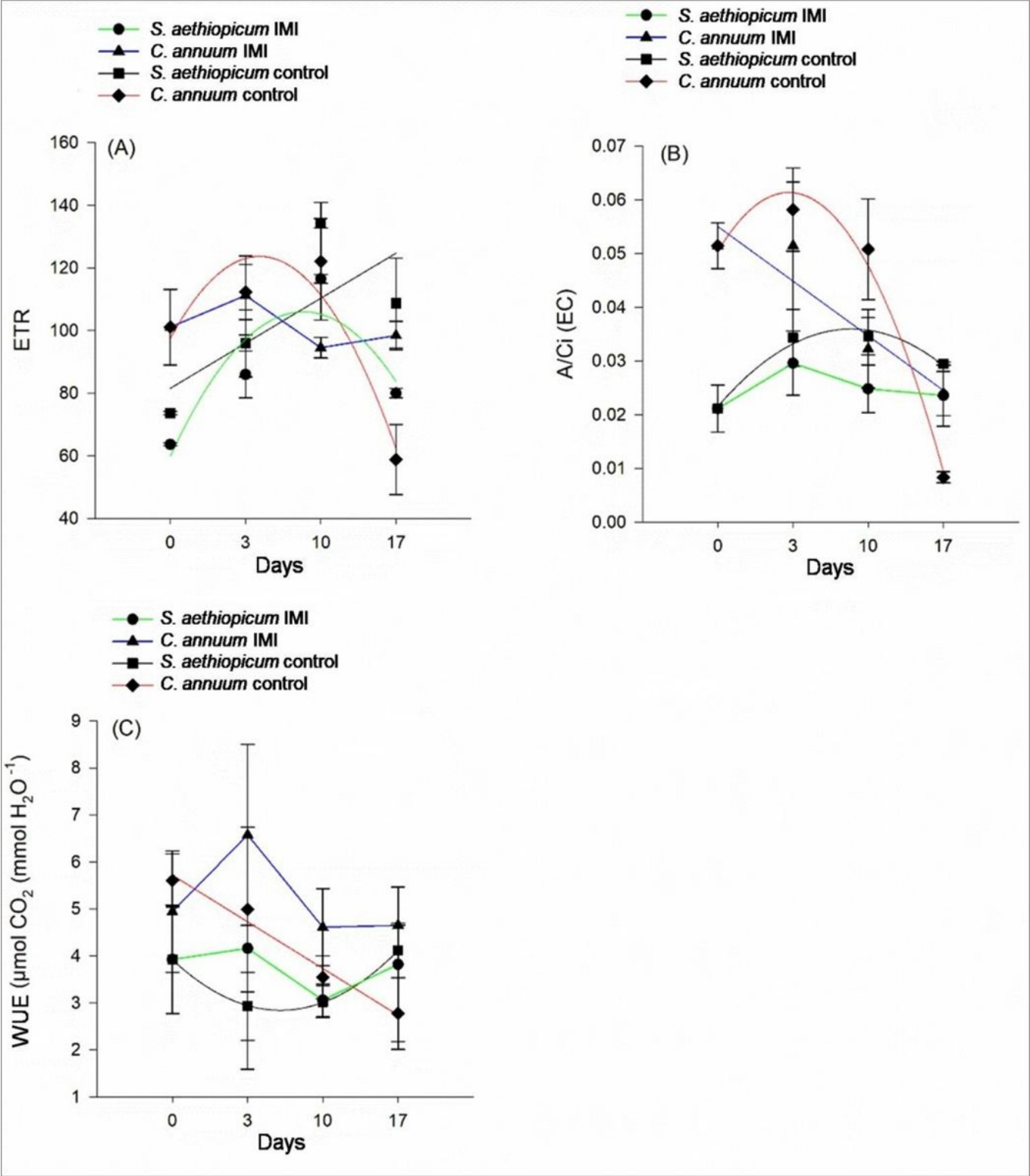


Figure 3

Graphical representation of the effect of imidacloprid (IMI) on gas exchange indices of *Solanum aethiopicum* and *Capsicum annuum* plants throughout the experimental period. *ETR*: electron transport rate; *A/C*: instantaneous carboxylation efficiency; *WUE*: water use efficiency.

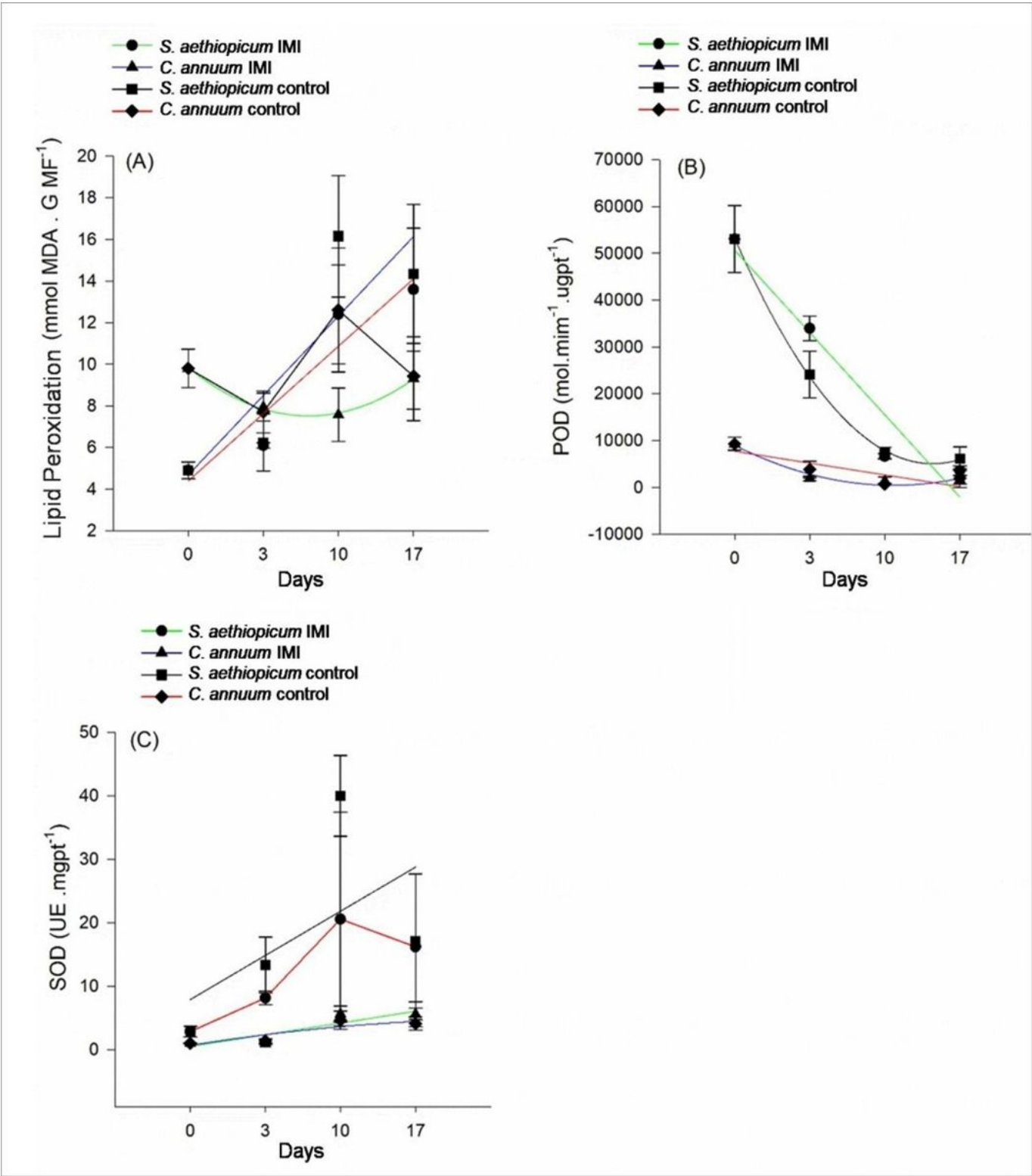


Figure 4

Graphical representation of the effect of imidacloprid (IMI) on the activity of (A) lipid peroxidation, and of oxidative enzymes (B) peroxidase (POD) and (C) superoxide dismutase (SOD) in *Solanum aethiopicum* and *Capsicum annuum* plants throughout the experimental period.

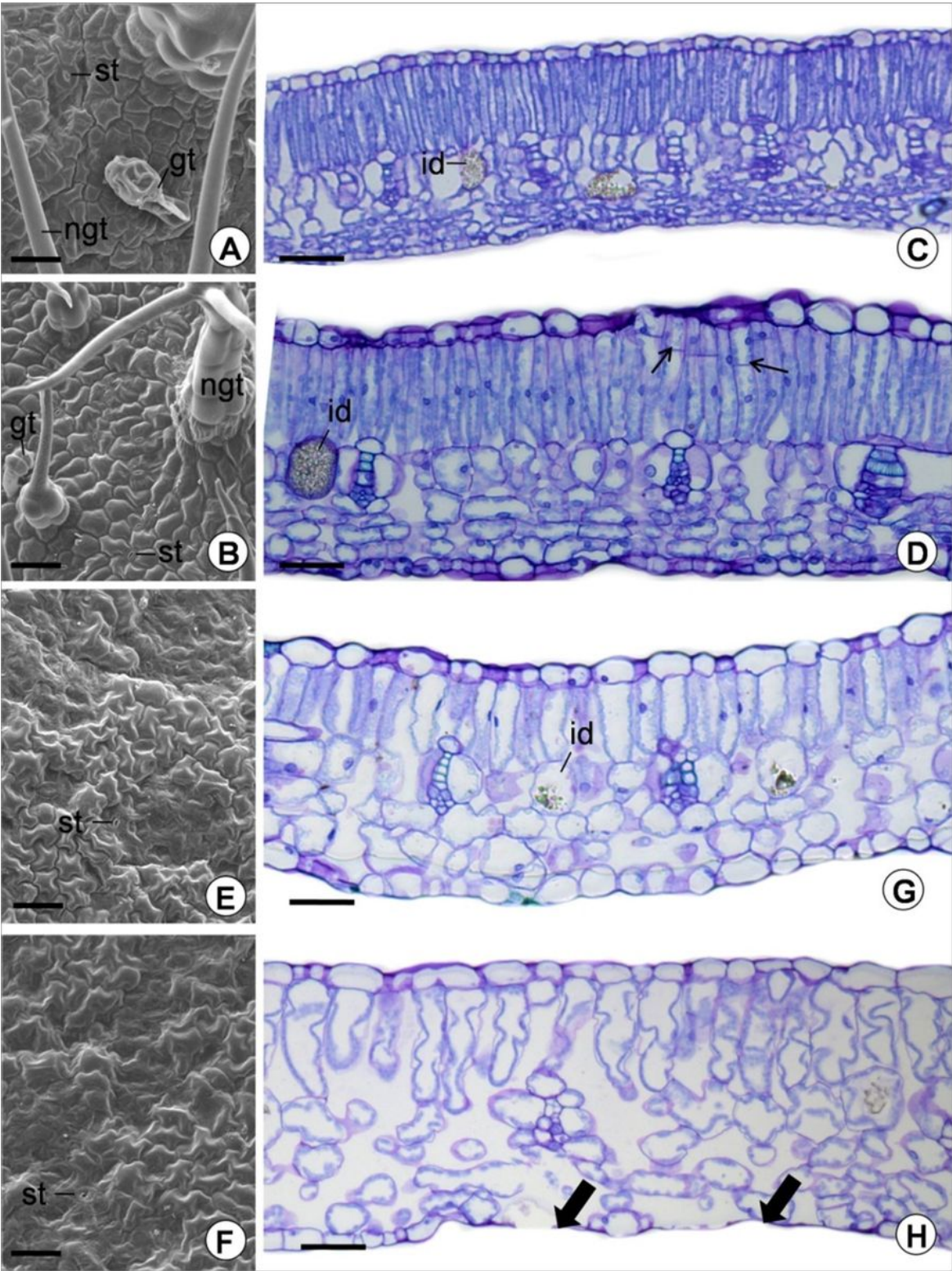


Figure 5

Scanning electron micrographs of the (A, B, E, F) adaxial surface and (C, D, G, H) photomicrographs of cross sections of the leaf blade of (A–D) *Solanum aethiopicum* and (E–H) *Capsicum annuum*. (A, C, E, G) Control plants. (B, D, F, H)imidacloprid-treated plants. st: stomata; id: idioblast; gt: glandular trichome; ng: non-glandular trichome; thin arrows: periclinal division; thick arrows: epidermis discontinuity in cross section caused by irregular relief on the leaf surface. (A, B, E, F) Scalebar =150 μm ; (C, D, G, H) Scale bar =100 μm .

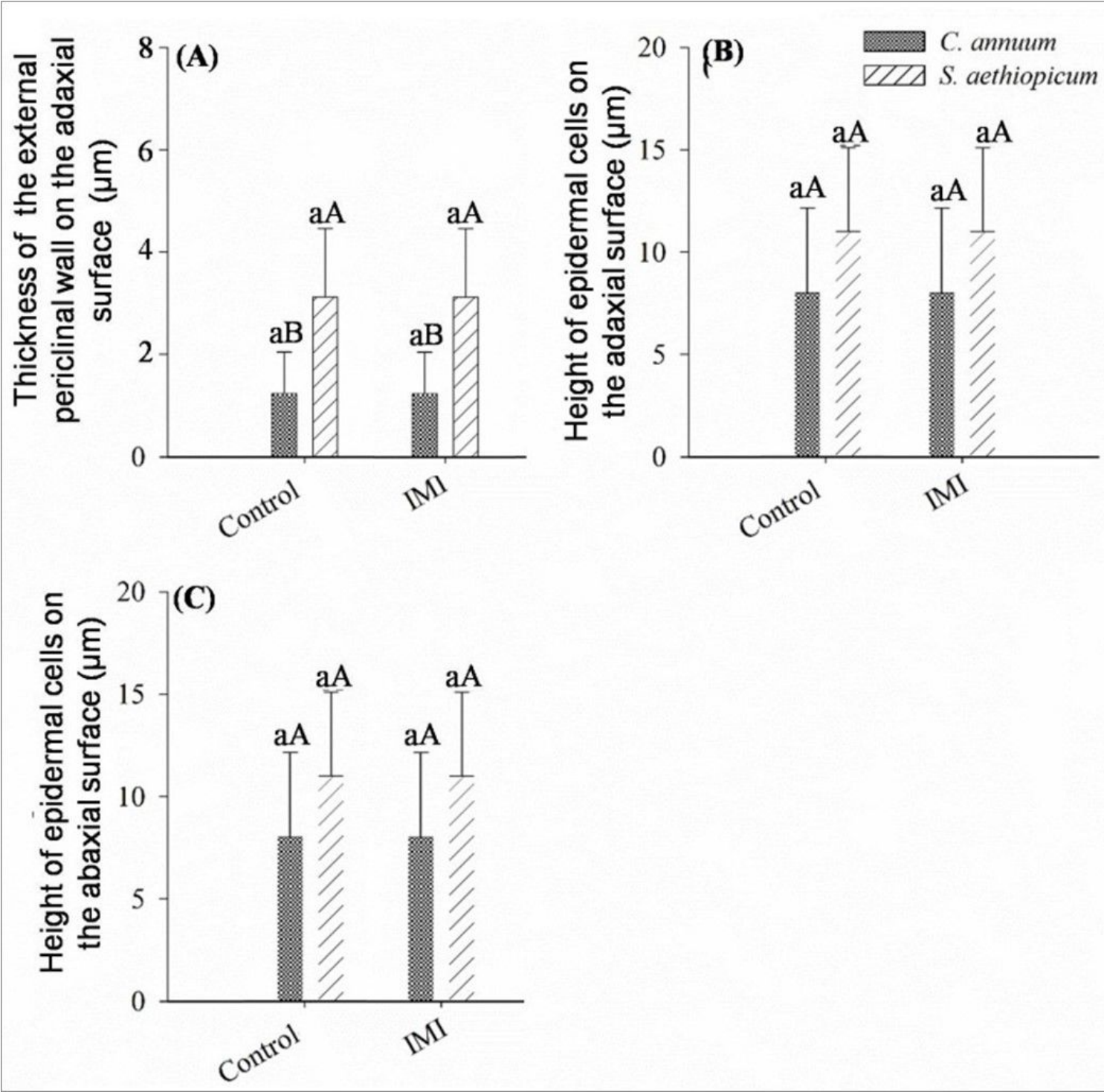


Figure 6

Morphometric data on *Solanum aethiopicum* and *Capsicum annuum* plants treated with imidacloprid (IMI) and control plants. Lower case letters indicate comparison between treatments and capital letters indicate comparison between species. Different letters indicate significant differences using Tukey's test.

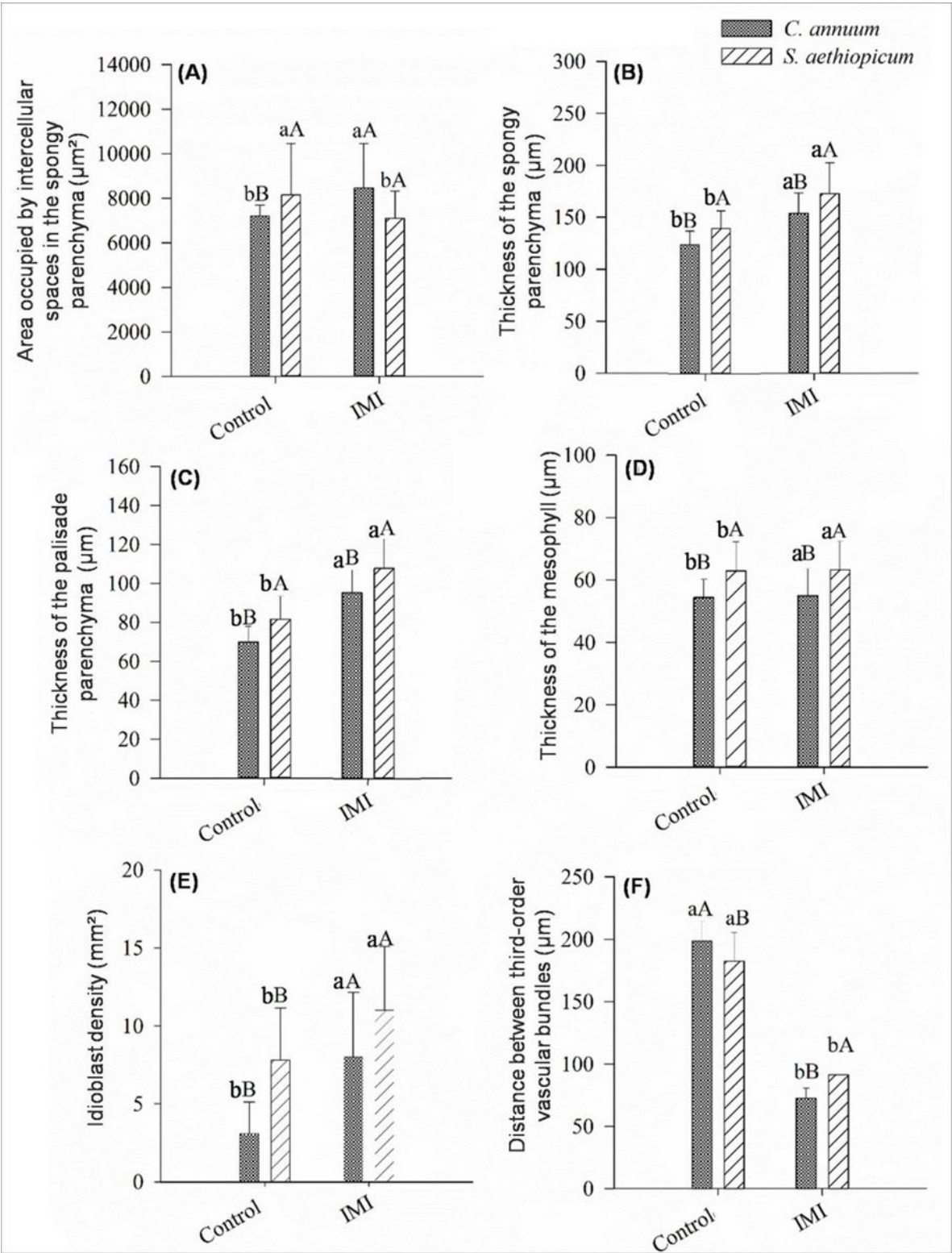


Figure 7

Morphometric data of *Solanum aethiopicum* and *Capsicum annuum* plants treated with imidacloprid (IMI) and control plants. Lowercase letters indicate comparison between treatments and capital letters indicate comparison between species. Different letters indicate significant differences using Tukey's test.

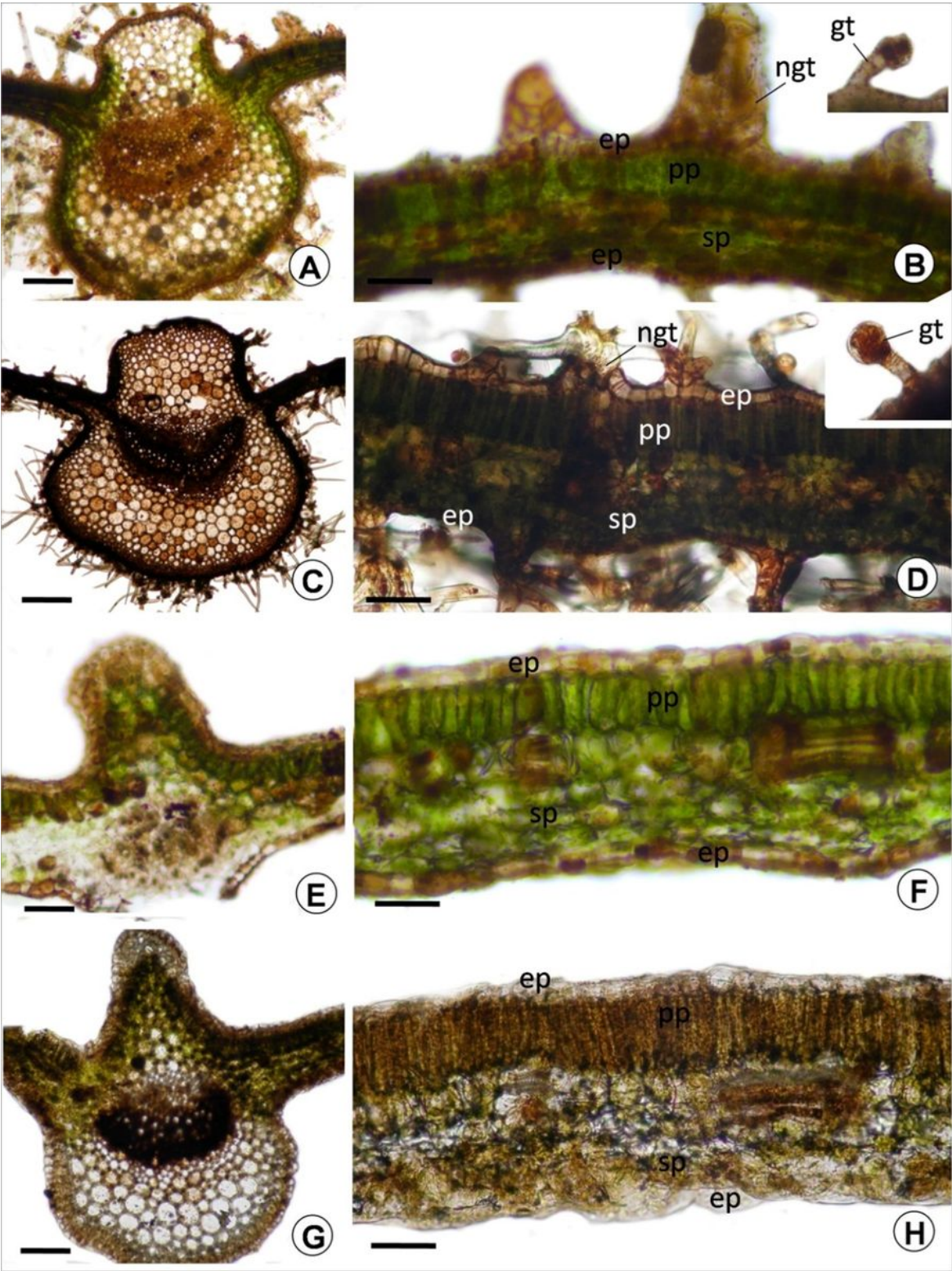


Figure 8

Photomicrographs of cross sections of (A–D) *Solanum aethiopicum* and (E–H) *Capsicum annuum* leaves treated with 3,3-diaminobenzidine (DAB) for reactive oxygen species (ROS) detection. (A, B, E, F) Control group plants. (C, D, G, H) Plants treated with imidacloprid. ep: epidermis; sp: spongy parenchyma; pp: palisade parenchyma; gt: glandular trichomes; ng: non-glandular trichomes. (A, C, G) Scale bars = 250 μ m; (B, D, E, F, H) Scale bars = 100 μ m.

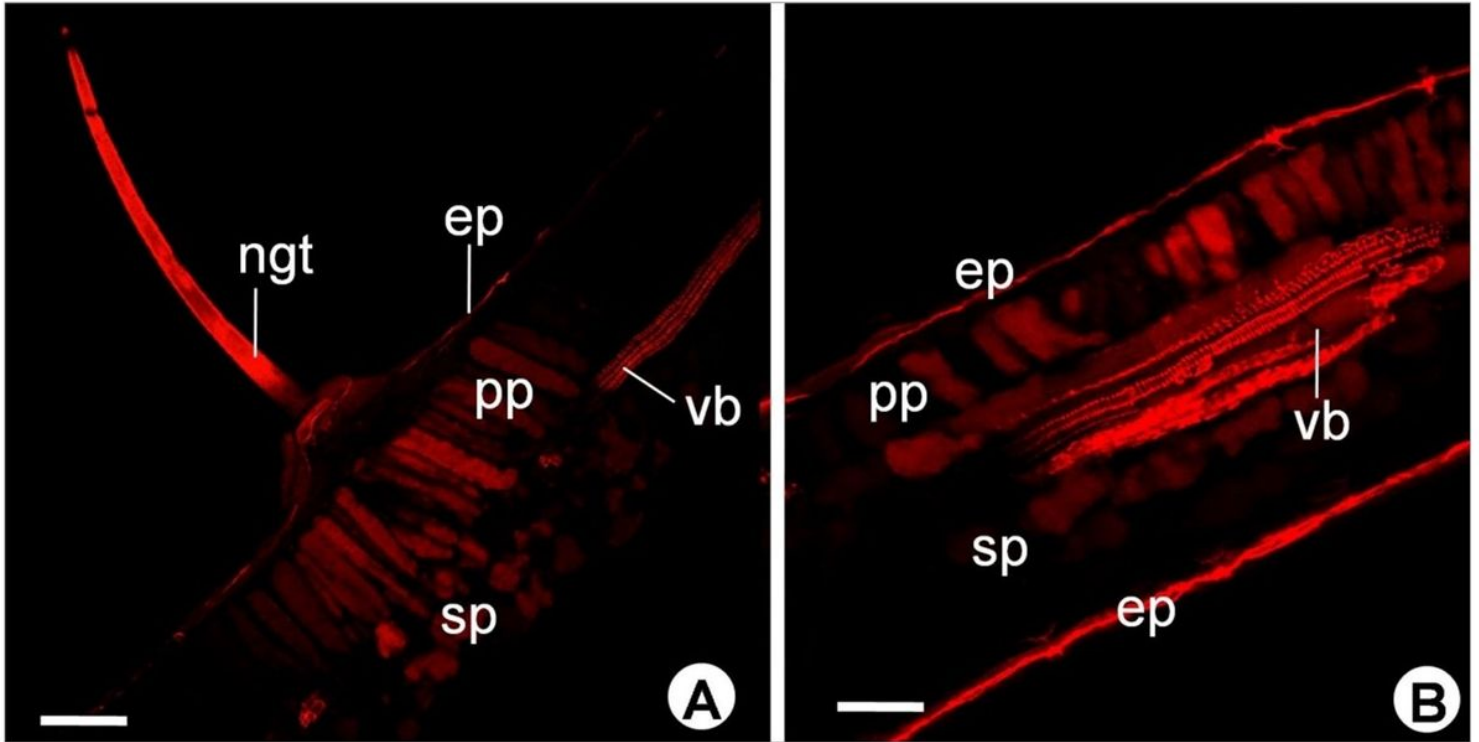


Figure 9

Confocal photomicrographs of leaves treated with 1% aqueous Safranin. (A) *Solanum aethiopicum*. (B) *Capsicum annuum*. ep: epidermis; vb: vascular bundle; sp: spongy parenchyma; pp: palisade parenchyma; ng: non-glandular trichome. Scale bars = 50 μ m.