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Responses of calcium-supplied Murcott IAC 221 tangor plants to *Alternaria alternata* infection

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Abstract - Alternaria brown spot - ABS (*Alternaria alternata*) causes lesions on fruits, leaves and branches of mandarins. The cost of fungicide application is high and alternative managements for the control need to be studied. We aimed to evaluate calcium (Ca) nutrition to mitigate the effects of ABS. Murcott IAC 221 tangor plants were nourished with calcium nitrate at three different concentrations (30, 150 and 300 mg Ca L^{-1}), and the N content was standardized in the nutrient solution of the treatments (245 mg L^{-1} of N) with ammonium nitrate. *In vitro* and *in vivo* tests were installed, evaluating the severity and area under the disease progress curve (AUDPC), total proteins, peroxidase (POX), catalase (CAT), superoxide dismutase (SOD) and hydrogen peroxide (H_2O_2), during four times. In all evaluations a negative correlation was observed between leaf Ca content and the severity and AUDPC of ABS. On the other hand, there was a positive correlation between N and the occurrence of the disease. In the treatment with the lowest concentration of calcium there was accumulation of H_2O_2 and hypersensitivity reaction (HR), with low detoxification of H_2O_2 by CAT. There is potential for use of calcium supply to plants of Murcott IAC 221 tangor as an alternative management against the fungus *A. alternata*.

Index Terms: alternaria brown spot; mandarins, nutritional management.

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Respostas de plantas de tangor Murcott IAC 221, supridas com cálcio, à infecção por *Alternaria alternata*

Resumo – A mancha marrom de alternária – MMA (*Alternaria alternata*) causa lesões em frutos, folhas e ramos de tangerinas. O custo da aplicação de fungicidas é alto, e manejos alternativos para o controle precisam ser estudados. Objetivou-se avaliar a nutrição com cálcio (Ca), para mitigar os efeitos da MMA. Plantas de tangor Murcott IAC 221 foram nutridas com nitrato de cálcio em três concentrações diferentes (30; 150 e 300 mg Ca L^{-1}), sendo o teor de N padronizado na solução nutritiva dos tratamentos (245 mg L^{-1} de N) com nitrato de amônia. Testes *in vitro* e *in vivo* foram instalados, avaliando-se a severidade e a área abaixo da curva de progresso da doença (AUDPC), proteínas totais, peroxidase (POX), catalase (CAT), superóxido dismutase (SOD) e peróxido de hidrogênio (H_2O_2), durante quatro tempos. Em todas as avaliações, observou-se correlação negativa entre o teor foliar de Ca e a severidade e AUDPC da MMA. Por outro lado, houve correlação positiva entre N e a ocorrência da doença. No tratamento com a menor concentração de cálcio, houve acúmulo de H_2O_2 e reação de hipersensibilidade (RH), com baixa detoxificação de H_2O_2 pela CAT. Há potencial para uso do suprimento com cálcio às plantas de tangor Murcott IAC 221 como manejo alternativo contra fungo o *A. alternata*.

Termos para indexação: mancha marrom de alternária; tangerinas, manejo nutricional.

Introduction

Alternaria brown spot (ABS) is caused by the fungus *Alternaria alternata* f.sp. *citri* (WOUDENBERG *et al.*, 2015), which produces a host selective toxin (*HST*), whose susceptibility is restricted to mandarins and some of their hybrids (YANG et al., 2016). Because most mandarins varieties are susceptible to ABS, the producer demands many applications of fungicides for its control, around 12 to 18 applications in the year, increasingly increasing the cost of production (AZEVEDO et al., 2010). Thus, the growing concern with pathogen resistance and the toxicity of fungicides has raised the need to seek genetic improvement of these varieties, as well as studies of alternative means of managing the disease.

Calcium (Ca) is an extremely important nutrient for the mineral nutrition of citrus, being directly linked to the quality of the fruit and in plant defense responses to phytopathogens (PETENÁ et al., 2016). In this way, the amount of Ca applied can affect both the mechanical and physiological resistance of the plant to diseases, contributing to the final structuring of the tissue and the stability of biomembranes. With Ca deficit, there is an increase in the production of sugars or other low molecular weight compounds in the apoplasm, favoring infection by phytopathogens (ALBERTS et al., 2011).

A large number of local and systemic plant defense mechanisms are activated in response to pathogen infection. Resistance exerted by R genes is usually accompanied by the rapid release of reactive oxygen species (ROS). The production of ROS is required by another component of the defense response, the hypersensitivity response (HR), where there is a kind of programmed cell death, limiting the development and infection by the pathogen (GLAZEBROOK, 2005). The ROS have a counterbalanced and balanced activity by the activity of endogenous antioxidant agents, such as the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) (ANDRADE et al., 2018).

During HR a series of enzymes are involved in the oxidative metabolism of the host, such as the NADPH-oxidase complex, forming the superoxide radical (O_{2}-), superoxide dismutase which transforms the superoxide radical into hydrogen peroxide (H_2O_2) and catalase, responsible for converting hydrogen peroxide into water and O_2 (HEISER and OSSWALD, 2008). During the process of elimination of hydrogen peroxide, the presence of peroxidases can be identified, as a substrate for synthesis of lignin or other compounds (PASSARDI et al., 2005), resulting in the formation of physical barriers at the site of infection, such as the formation of callose, papillae, cell wall thickening or even the formation of compounds toxic to the pathogen, such as phytoalexins and peroxidases (PASCHOLATI; DALIO, 2018). On the other hand, as well as plants, some necrotrophic pathogens like *A. alternata,* can synthesize catalase, to protect themselves from ROS and allowing their infection in the host (YANG; CHUNG, 2012).

The objective of this work was to evaluate the interaction of nutrients (nitrogen and calcium) in the induction of resistance to the fungus *A. alternata in* Murcott IAC 221 tangor and to analyze the accumulation of enzymes in response to the action of ABS.

Material and methods

Installation of seedlings

Plants of Murcott IAC 221 tangor grafted onto rangpur lime (*C. limonia* Osbeck) received the same nutritional treatment for six months after grafting, applying fortnightly 250 ml of fertilizer solution, according to Hippler et al. (2015). After six months in the nursery, the plants were transplanted and maintained in the greenhouse, in 20 L capacity pots with Pinus bark substrate, and grown under differential supply regime with three levels of soluble Ca, supplied via fertigation, varying the composition of the nutrient solution with ammonium nitrate (AN) and calcium nitrate (CN), keeping equal the levels of other nutrients: in mg L^{-1}, Ca (30, 150 or 300), P (31.2), Mg (56.8), S (78.4), B (0.49), Cu (2.0), Fe (3.0), Mn (2.54),

Mo (0.03) and Zn (1.68). The plants were irrigated five times a day, with three localized fertigation and two with only sprinkler water. The fertirrigation was 120 L per year per plant (2.5 L per week per plant). The treatments were: 30, 150 and 300 mg L^{-1} of Ca, supplied respectively, by the doses (g 1000 L^{-1}): 176.5; 882 and 1765 of CN, from the fertigation management, using nutritive solution containing 245 mg L^{-1} of N when combining the application of AN with that of CN. The plants were maintained in this fertigation management for 6 months. The plants were trained by pruning, with four branches per plant during growth, and the tips were pruned to induce new shoots, allowing the leaves to grow to a size of approximately 2-3 cm.

Isolation of *Alternaria alternata* fungus and preparation of inoculum

For the *in vitro* and *in vivo* inoculation experiments, the isolate of *A. alternata* was obtained from typical lesions of the disease on highly susceptible Murcott IAC 221 tangor fruits collected in the field from the Sylvio Moreira Citrus Growing Center (CCSM) of the Agronomic Institute (IAC), located in Cordeirópolis, SP, where the fungus is endemic. To obtain the monospore isolate, the methodology described by Peever et al. (1999) with modifications was used (AZEVEDO et al., 2010).

The preparation of the inoculum was performed by transferring inverted discs of mycelium of the fungus (~8 mm Ø) to Petri dishes, and kept in BOD for seven days, with a photoperiod of 12 hours, at approximately 27ºC, according to the methodology of Canihos et al. (1999). After mycelial growth, 10 ml of distilled water was added on the surface of the plate, and with the help of a sterile Drigalski loop, the conidia were removed from the surface, and then the concentration was adjusted to 10^5 conidia mL^{-1}, with the help of the Neubauer chamber.

In vitro and *in vivo* assays

The inoculation trials were performed in 2019 and 2020 in an entirely randomized design with seven repetitions of each treatment. In the *in vitro* inoculation assay, each repetition of the experiment consisted of a Petri dish containing three young leaves of Murcott IAC 221 tangor (detached), with approximately 2-3 cm in length and around two weeks old. The detached leaves were washed in running water and placed in Petri dishes, containing a layer of filter paper and a small portion of moistened cotton to maintain humidity. In the *in vivo* experiment, four leaves from three different branches of the same plant were evaluated in seven repetitions of Murcott IAC 221 tangor plants.

Inoculation was performed according to Canihos et al. (1999) with a suspension of *A. alternata* spores (10^5 conidia mL^{-1}) using a manual sprayer, applying 1 ml on the abaxial side of the leaves. The *in vitro* experiment plates were maintained in BOD at 27ºC with a 12 hour photoperiod for five days. The plants of the *in vivo* experiment were maintained in an acclimatized growth chamber, with controlled relative humidity (80 ± 5%), temperature (27 ± 1 ºC) and photoperiod of 12 hours until the end of the evaluation.

Evaluations of ABS severity and area under the disease progress curve (AUDPC)

The evaluations of the lesions caused by the fungus, for severity evaluation, in the in *vitro* and *in vivo* assay occurred in 24, 48, 72, 96 and 120 hours after inoculation, through the observation of the presence of typical symptoms of the disease and, subsequently in determinations of the injured area (% of the leaf taken by the disease) as described by Martelli et al. (2016). Using the severity data the area under the disease progress curve (AUDPC) was calculated, which is expressed by plotting the proportion of disease in percentage using the formula of Shaner and Finney (1977).

Protein contents and enzymatic activities in leaves of Murcott IAC 221 tangor after inoculation, *in vivo*, with *A. alternata* Evaluations of total protein content and enzymatic activities were performed on Murcott IAC 221 tangor leaves after inoculation, in *vivo*, with *A. alternata*, in 2020. For this, after inoculation *in vivo* samples were collected at 24, 48 and 72 hours after inoculation, except for the time 0 (zero) that was performed before the leaves were inoculated. The samples immediately after collection were wrapped in aluminum foil, immersed in liquid nitrogen and stored in an ultrafreezer at -80 ºC. Total protein extraction was performed according to Felipini et al. (2016) with modifications. Leaves (200 mg) were macerated in the presence of liquid nitrogen and homogenized in 1.5 mL of 100 mM phosphate buffer (pH 7.0), polyvinylpyrrolidone (0.5 % m/v) and ethylenediaminetetraacetic acid (EDTA 1 mM). Homogenates were arranged in 2.0 mL capacity microtubes and centrifuged at 20,000 *g* for 30 min at 4 ºC. The supernatant (protein extract) was collected and stored at -80 ºC until the moment of evaluation. Total protein determination was performed by the Bradford method (1976).

Superoxide dismutase (SOD) activity was quantified according to Giannopolitis and Ries (1977). The results were expressed as U SOD mg $protein^{-1}$, one unit of SOD being the amount of enzyme necessary to inhibit 50 % of the photoreduction of NBT to formazan. Peroxidases (POX) activity was measured according to Hammerschmidt *et al.* (1982). Results were expressed as optical density units at 470 nm per min per mg protein (OD. min^{-1} .mg $protein^{-1}$). Catalase (CAT) activity was quantified in a spectrophotometer according to Kraus et al. (1995) with modifications by Azevedo et al. (1998). The activity was calculated based on the extinction coefficient of H_2O_2 (39.4 $M.cm^{-2}$) and the results expressed as µmol.min^{-1}.mg protein $^{-1}$.

Quantification of hydrogen peroxide in the leaves

The quantification of hydrogen peroxide content in leaves was performed according to Hippler et. al (2015). The absorbance values were interpolated to a standard curve

prepared with known concentrations of hydrogen peroxide. The result was expressed as µmol g^{-1} of fresh matter.

Nutrient analysis in the leaves of Murcott IAC 221 tangor

For foliar analysis of N and Ca, about 100 g of new leaves (2-4 cm long) from all branches of the crown from ten plants per treatment were collected in 2019 and 2020. The leaves were thoroughly washed in 0.08% detergent solution ($v.v^{-1}$) and rinsed twice in distilled water, then were oven dried (60 °C). After 72h, a manual mill was used to grind 200 mg of dry mass. The material was sent to the Soil Fertility Laboratory of the Agronomic Institute (IAC, Campinas/SP) for analysis according to Bataglia et al. (1983).

Data analysis

The values of severity, AUDPC and enzyme activity were subjected to variance analysis and comparison of means by the Tukey test at 5% probability using the *software* SISVAR 4.5 (FERREIRA, 2011). The data of severity, AUDPC and nutrients (calcium and nitrogen) were subjected to Pearson's correlation analysis using the t-test at 5% probability level. The data for each variable were analyzed using the GLM Procedure of the SAS software (version 6.12).

Results and Discussion

Severity and area under the disease progress curve (AUDPC) assessments ABS

Typical symptoms of ABS on detached leaves were observed 24 hours after *in vitro* inoculation in all treatments, since the variety under study (Murcott IAC 221 tangor) is highly susceptible to the fungus *A. alternata* in the field and in tests with detached leaves (AZEVEDO et al., 2010; BASTIANEL et al., 2014; MICHIELIN et al., 2016). These symptoms were characterized by small brown and black spots, with a chlorotic halo and varying in size on the leaves. These spots, with time of evaluation, expanded creating necrotic areas in the leaf tissues due to the action of ACT toxin, as described by some authors (KOHMOTO et al., 1993; AKIMITSU et al., 2003).

In the two years of evaluation of the *in vitro* experiment (Figure 1 A-D), it was observed that the plants with a nutritional regime with a higher calcium content (300 mg L^{-1}) were more resistant to ABS. In AUDPC the treatments with 30 and 150 mg Ca L^{-1} , did not differ from each other in 2019 (Figure 1 B), but in the second year of evaluation, the three treatments differed from each other (Figure 1 D). Possibly, calcium being a structural and functional component of cell walls and middle lamellae, the treatment with the highest concentration of calcium showed greater tissue thickening, favoring the resistance of the plant to the pathogen. A similar result was observed by Petená et al. (2016), where the treatment with the highest concentration of calcium altered the cellular anatomy so that the thickening of the cells acted as a physical barrier to infection by the fungus *Colletothrichum acutatum*, which causes citrus post-bloom fruit drop.

In the first year of evaluation of the *in vivo* experiment it was possible to observe that the severity and the AUDPC of the treatment with 300 Ca mg L^{-1} (Figure 2 A and B) did not differ from the treatment that has 50% of the calcium dose (150 mg Ca L^{-1}), evidencing that for *in vivo* tests the two doses allowed the development of the disease equally. The results of the second year of evaluation (2020) *in vivo*, indicated that the treatment that provided greater resistance to the fungus *A. alternata,* causing ABS, was with higher dose of calcium (Figure 2 C and 2 D), differing statistically from the others in severity (%), after 96 hours of inoculation, and AUDPC. One of the reasons that may have caused greater disease growth in the plants treated with the lowest dose of Ca (Figure 3) is that the application of nitrogen without the presence of calcium led to the development of cells with unrestrained and irregular elongation, making them disuniform in size and shape and, consequently, forming loose tissues, as observed by Petená et al. (2016) in

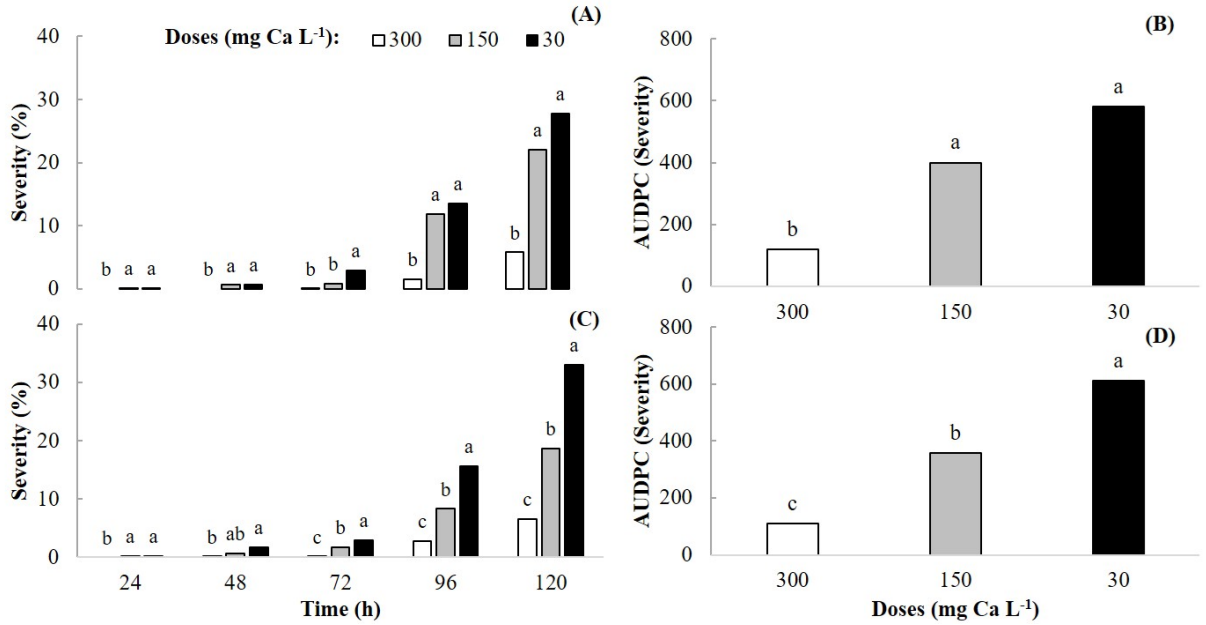

Figure 1 - Severity of symptoms caused by *Alternaria alternata* at 24,48, 72, 96 and 120 hours after *in vitro* inoculation (A) and (C) and area under the disease progress curve (AUDPC) - (B) and (D), in leaves of Murcott IAC 221 tangor plants, with differential calcium regime (30, 150 and 300 mg Ca L^{-1}), in the years 2019 and 2020, respectively (Cordeirópolis, Sao Paulo State, Brazil). NC = calcium nitrate and NA = ammonium nitrate. (*) means followed by the same letter, do not differ (Tukey, 5%).

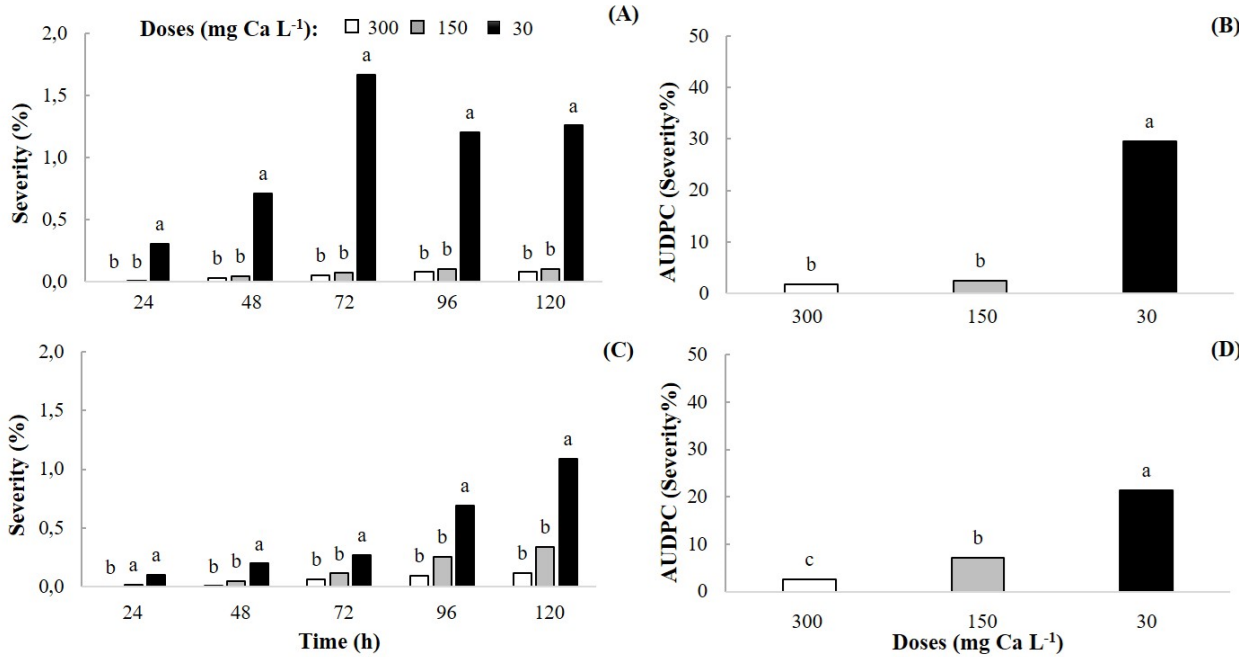

Figure 2 - Severity of symptoms caused by *A. alternata* at 24, 48, 72, 96 and 120 after inoculation, *in vivo,* in the years 2019 (A) and 2020 (C) and area under the disease progress curve (AUDPC) in the years 2019 (B) and 2020 (D), in leaves of plants of Murcott IAC 221 tangor, with differential calcium regime (30, 150 and 300 mg Ca L^{-1}), (Cordeirópolis, Sao Paulo State, Brazil). CN = calcium nitrate and AN = ammonium nitrate. (*) means followed by the same letter do not differ (Tukey, 5%).

Valencia orange leaves. The treatments with doses of calcium, on the other hand, probably formed cell walls and medium lamellae with greater density and thickness, making the tissues compact (TAIZ; ZEIGER, 2006). The main route of penetration of *A. alternata* is directly through the leaf surface, so with the greater thickening of these barriers, it favors greater mechanical resistance to the attack of the fungus (STUART et al., 2009).

Figure 3 - Symptoms of alternaria brown spot (ABS) in leaves of Murcott IAC 221 tangor plants supplied with the highest (300 mg Ca L^{-1}) and lowest dose calcium - 30 mg Ca L^{-1} (A) and ammonium nitrate (B), after 120h of inoculation *in vivo*. (Cordeirópolis, Sao Paulo State, Brazil, 2019). Red arrow indicates typical symptoms of ABS on Murcott IAC 221 tangor leaves.

Protein contents and enzymatic activities in leaves of Murcott IAC 221 tangor after inoculation, *in vivo*, with *A. alternata*

After inoculation *in vivo,* the quantity of total proteins and enzymatic activity produced by the plant *in* response to infection by the pathogen (*A. alternata*) was evaluated. In the results of total proteins, it was observed that the treatment with the highest dose of calcium (300 mg Ca L^{-1}) presented a greater quantity of proteins than the other treatments (Figure 4 A). When the production of antioxidant enzymes like SOD was evaluated, the treatment with the lowest dose of calcium (30 mg L^{-1}) had the highest production 24 hours after inoculation (Figure 4 B) and the treatment with 150 mg Ca L^{-1}, after 72 hours.

The highest peroxidase activity was observed 48 and 72 hours after inoculation of the pathogen in the treatment with 30 mg Ca L^{-1} (Figure 4 C). Higher catalase activity was observed in the treatment with the highest dose of Ca (300 mg Ca L^{-1}), 48 and 72 hours after inoculation in relation to the other treatments (Figure 4 D), being 85% higher than the treatment with 150 mg Ca L^{-1}, 72 hours after inoculation.

Higher concentrations of hydrogen peroxide were observed in the treatment with the highest dose of Ca at 24, 48 and 72 hours after inoculation, when compared with the treatments with lower doses of Ca (Figure 4 E). During the evaluation times, no differences in peroxide concentration were noted in the treatments with 30 and 150 mg Ca L^{-1} (Figure 4 E).

Through the results obtained, it was possible to observe that the alterations in the enzymatic activity occurred from 24 hours after inoculation, due to the beginning of the infection process of the fungus *A. alternata*. Renaud et al., (2005) and Timmer et al., (2003) observed that 24 hours after inoculation occurs the germination of spores and the beginning of the infectious process of *A. alternata* in an environment with adequate temperature and humidity, where the production of ACT toxin occurs, specific for mandarin and its hybrids, causing necrosis of the infected site and spreading rapidly through the tissues of the leaf or fruit.

After the recognition of the pathogen by the plant occurs the production and accumulation of reactive oxygen species (ROS), which after infection, serves as one of several barriers during the colonization of the

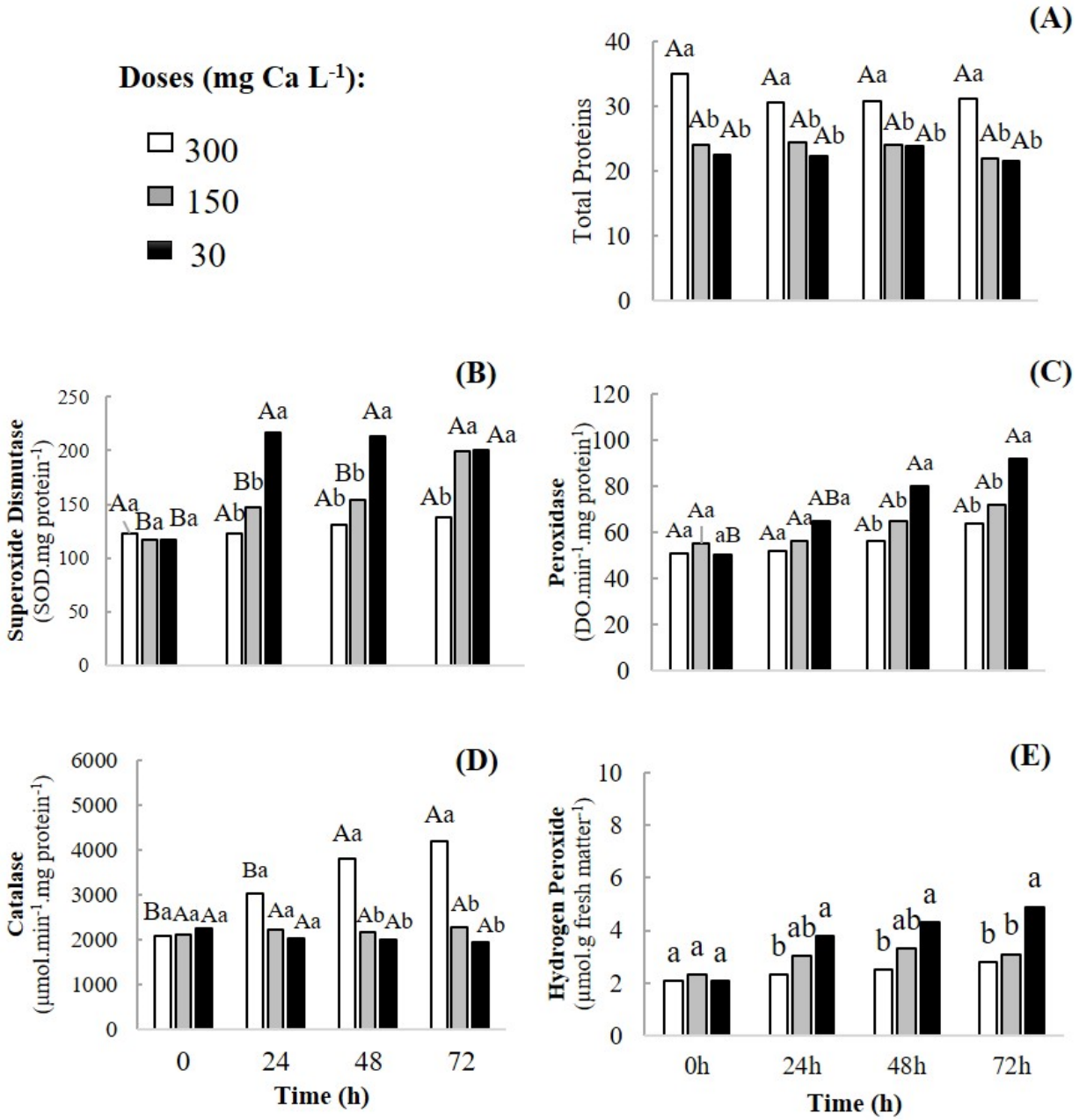

Figure 4 - Quantification of total proteins (A), superoxide dismutase activity (B), peroxidases activity (C), catalases activity (D), hydrogen peroxide content (E) in Murcott IAC 221 leaves from plants supplied with differential regimen with calcium (30, 150 and 300 mg Ca L^{-1}), at different hours after inoculation. Detached leaves were sprayed with 10^5 spores. mL^{-1} spores. mL^{-1} of *Alternaria alternata* (*). Means followed by the same letter (capital letters differentiating between times of the same treatment and iglower-case letters differentiating between treatments within the same time) did not differ (Tukey, 5%). (Cordeirópolis, Sao Paulo State, Brazil, 2021).

pathogen, mainly during oxidative stress (GREENBERG and YAO, 2004). Enzymes arising from the activity of the NADPH-oxidases enzyme complex, precursors of ROS, result in electron transfer and formation of superoxide radical (O^-), resulting in lipid peroxidation or be transformed into hydrogen peroxide (H_2O_2) by the action of superoxide dismutase (SOD) (ROUT and SAHOO, 2013; GRATÃO et al., 2015). Thus, the treatment with the highest dose of Ca was able to react to the infection of the pathogen, maintaining the synthesis of SOD at stable values, due to the increased concentration of the superoxide radical in the plants of this treatment.

The opposite is also observed in the treatment with the lowest dose of Ca, where there was a greater concentration of hydrogen peroxide. This concentration at high levels in response to plant stress becomes toxic to the cell, producing the hydroxyl radical (OH^-), one of the most reactive forms, caus ing damage to the membrane (CAPALDI et al., 2015; HIPPLER et al., 2015). In response to this increase in ROS, plants require an efficient antioxidant system to eliminate the superoxide radical (O_2), requiring greater production of SOD to be realized, which corroborates the results found for the treatment with the lowest dose of Ca (300 mg Ca L^{-1}).

In the metabolism of ROS occurs the detoxification of hydrogen peroxide by the action of catalase (CAT), enzyme that transforms H_2O_2 into H_2O and O_2 (RESENDE et al., 2003). Thus, in the treatment with the lowest dose of calcium (30 mg Ca L^{-1}), low CAT activity was obtained during the studied times, and followed by the accumulation of hydrogen peroxide in the treatment, the hypersensitivity reaction (HR) occurred.

Hypersensitivity reaction is a plant defense reaction to attack by microorganisms, which leads to programmed cell death. HR plays a vital role in plant defense against biotrophic or saprophytic pathogens, however, HR is not effective against necrotrophic pathogens, since these pathogens acquire their nutrients exclusively from dead tissues (DAUB et al., 2005). For the disease to occur, it is necessary the elimination of ROS using its NADPH-oxidases complex, that is, the hypersensitivity reaction can make the phenotype susceptible or resistant, according to the type of fungus (YANG and CHUNG, 2012).

Analysis of nutrients in leaves of Murcott IAC 221 tangor and the severity and AUDPC of the disease

Using Pearson's correlation it was possible to observe significant correlations between the severity of the disease and the AUDPC with the content of Ca and N in the leaves in 2019 and 2020, both in the *in vivo* and *in* vitro experiments (Table 1). Negative correlation was found for Ca and positive for N, indicating that the higher the calcium content in the leaf, the lower were the disease severity indices *in vitro* and *in vivo*. Significant positive correlations were found for N, both for severity and AUDPC, indicating that the greater the presence of N in the leaves of the plants, the greater the susceptibility of the plant to infection by *A. alternata*.

The resistance of plants to pests and diseases may be diminished or increased by the effect of mineral nutrition on anatomical structures, for example: thinner epidermal cells and cuticles, a cell wall with a lower degree of suberization and lignification. Among the macronutrients, Ca deficiency is one of those that provokes the greatest structural and biochemical changes, making the plants more susceptible to pathogen attack. Excess N can also make the plants less resistant to fungal infections by reducing the synthesis of phenolic compounds like lignin (DUFFY; DÉFAGO, 1999; SILVEIRA et al., 2003).

Table 1 - Pearson's correlation matrix between severity of alternaria brown spot *in vitro* and *in vivo*, area under the disease progress curve (AUDPC) *in vivo* and *in vitro* and levels of calcium and nitrogen present in the plants, in the years 2019 and 2020 (Cordeirópolis/SP).

Parameters/Nutrients	Pearson correlation coefficients			
	2019		2020	
	Calcium	Nitrogen	Calcium	Nitrogen
Severity *in vitro*	-0.50*	0.85*	-0.51*	0.76*
AACPD *in vitro*	-0.64*	0.83*	-0.69*	0.80*
Severity *in vivo*	-0.93*	0.51*	-0.90*	0.53*
AACPD *in vivo*	-0.95*	0.50*	-0.92*	0.51*

*Pearson correlation coefficients followed by an asterisk are significant (P=0.05)

Conclusion

The treatment with the highest dose of calcium (300 mg Ca L^{-1}) induces in the plants of Murcott IAC 221 tangor greater resistance to the fungus *A. alternata*, the cause of alternaria brown spot. In the treatment with the lowest dose of Ca (30 mg Ca L^{-1}) occurs the accumulation of hydrogen peroxide and hypersensitivity reaction (HR), with low detoxification of hydrogen peroxide by catalase (CAT). In turn, the highest dose of calcium leads to an increase in the levels of CAT, with no accumulation of hydrogen peroxide and, consequently, HR. Plants with higher levels of Ca present a lower severity of the disease, consequently causing less stress to the plant and less release of enzymes.

Acknowledgements

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References

AKIMITSU, K.; PEEVER, T.L.; TIMMER, L.W. Molecular, ecological and evolutionary approaches to understanding Alternaria diseases of citrus. **Molecular Plant Pathology**, Sheffield, v.4, p.435-6, 2003.

ALBERTS, B.; BRAY, D.; HOPKIN, K.; JOHNSON, A.; LEWIS, J.; RAFF, M.; ROBERTS, K.; WALTER, P. **Fundamentos da biologia celular**. Porto Alegre: Artmed, 2011. v.3, 740 p.

ANDRADE, C.A.; SOUZA, K.R.D. de; OLIVEIRA S.M. de; SILVA D.M. da; ALVES J.D. Hydrogen peroxide promotes the tolerance of soybeans to waterlogging. **Scientia Horticulturae**, Amsterdam, v.232, p.40-5, 2018.

AZEVEDO, R.A.; ALAS, R.M.; SMITH, R.J.; LEA, P.J. Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation, in the leaves and roots of wild-type and catalase-deficient mutant of barley. **Physiologia Plantarum**, Oxford, v.104, p.280–292, 1998.

AZEVEDO, F.A.; POLYDORO, D.A.; BASTIANEL, M.; KUPPER, K.C.; STUART, R.M.; COSTA, F.P.; PIO, R.M. Resposta de diferentes genótipos de tangerinas e seus híbridos à inoculação in vitro e in vivo de Alternaria alternata. **Revista Brasileira de Fruticultura**, Jaboticabal, v.32, n.1, p.1-10, 2010.

BASTIANEL, M.; SIMONETTI, L.M.; SCHINOR, E.H.; GIORGI NETO, R.O.; DE NEGRI, J.D.; GOMES, D.N.; AZEVEDO, F.A. Avaliação do banco de germoplasma de mexericas com relação às características físico-químicas e suscetibilidade à mancha marrom de alternária. **Bragantia**, Campinas, v.73, p.23-31, 2014.

BATAGLIA, O.C.; FURLANI, A.M.C.; TEIXEIRA, J.P.F.; FURLANI, P.R.; GALLO, J.R. **Método de análise química de plantas**. Campinas: Instituto Agronômico, 1983. (Boletim técnico, 78).

CANIHOS; PEEVER, T.L.; TIMMER, L.W. Temperature, leaf wetness, and isolate effects on infection of Minneola tangelo leaves by *Alternaria* spp. **Plant Disease**, St Paul, v.83, p.429-33, 1999.

CAPALDI, F.R., GRATÃO, P.L., REIS, A.R., LIMA, L.W. & AZEVEDO, R.A. Sulfur metabolism and stress defense responses in plants. **Tropical Plant Biology**, v.8, p.60-73, 2015.

DAUB, J.T.; HOFER, T.; CUTIVET, E.; DUPANLOUP, I.; QUINTANA-MURCI, L.; ROBINSON-RECHAVI, M.; EXCOFFIER L. Evidence for polygenic adaptation to pathogens in the human genome. **Molecular Biology and Evolution**, Laurence, v.30, p.1544-58, 2013.

DUFFY, B.K.; DÉFAGO, G. Macro-and microelement fertilizers influence the severity of Fusarium crown and root rot of tomato in soilless production system. **HortScience**, St Joseph, v.34, p.287-91, 1999.

FELIPINI, R.B.; BONETI, J.I.; KATSURAYAMA, Y.; NETO, A.C.R, VALEIRINHO, B.; MARASCHIN, M.; DI PIERO, R.M. Apple scab control and activation of plant defence responses using potassium phosphite and chitosan. **European Journal of Plant Pathology**, Dordrecht, v.145, p.929-39, 2016.

FERREIRA, D.F. Sisvar: a computer statistical analysis system. **Ciência e Agrotecnologia**, Lavras, v.35, p.1039-1042, 2011.

GIANNOPOLITIS C.N.; RIES S.K. Superoxide dismutases: I. Occurrence in higher plants. **Plant Physiology**, Lancaster, v.59, p.309-14, 1977.

GLAZEBROOK, J. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. **Phytopathology**, St Paul, v.43, p.205-27, 2005.

GRATÃO, P.L.; MONTEIRO, C.C.; TEZOTTO, T.; CARVALHO, R.F.; ALVES, L.R.; PERES, L.E.P.; AZEVEDO, R.A. Cadmium stress antioxidant responses and root-to-shoot communication in grafted tomato plants. **Biometals**, Oxford, v.28, p.803-16, 2015.

GREENBERG, J.T.; YAO, N. The role and regulation of programmed cell death in plant–pathogen interactions. **Microbiologia Celular**, Oxford, v.6, p.201-11, 2004.

HAMMERSCHMIDT R.; NUCLES E.M.; KUC J. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. **Physiological Plant Pathology**, London, v.20, p.73–82, 1982.

HEISER I.; OSSWALD W.F. Formação e função das espécies reativas de oxigênio nas interações planta-patógeno. *In*: PASCHOLATI S.F.; LEITE B.; STANGARLIN J.R.; CIA P. I**nteração planta patógeno**: fisiologia, bioquímica e biologia molecular. Piracicaba: FEALQ, 2008. v.3, p.627.

HIPPLER, F.W.R.; BOARETTO, R.M.; QUAGGIO, J.A.; BOARETTO, A.E.; ABREU-JUNIOR, C.H.; MATTOS-JR, D. Uptake and distribution of soil applied zinc by Citrus trees - addressing fertilizer use efficiency with Zn labelling. **PLoS One**, San Francisco, v.10, p.37-40, 2015.

KOHMOTO, K.; ITOH, Y.; SHIMOMURA, N.; KONDOH, Y.; OTANI, H.; KODAMA, M.; NAKATSUKA, S. Isolation and biological activities of two host-specific toxins from the tangerine pathotype of *Alternaria alternata*. **Phytopathology**, St Paul, v.83, p.495-502,1993.

KRAUS, T.E.; MCKERSIE, B.D.; FLETCHER, R.A. Paclobutrazol-induced tolerance of wheat leaves to Paraquat may involve increased antioxidant enzyme activity. **Journal of Plant Physiology**, Jena, v.145, p.570-6, 1995.

MARTELLI, I.B.; PACHECO, C.A.; BASTIANEL, M.; SCHINOR, E.H.; CONCEIÇÃO, P.M.; AZEVEDO, F.A. Diagrammatic scale for assessing foliar symptoms of alternaria brown spot in citrus. **Agronomy Science and Biotechnology**, Londrina, v.2, p.56-61, 2016.

MICHIELIN T.H.V.; CRISTOFANI-YALY M.; CAMPOS K.A.F.; SCHINOR E.H.; AZEVEDO F.A.; BASTIANEL M. Reação de híbridos de citros à inoculação com *Alternaria alternata*. **Summa Phytopathologica**, Botucatu, v.42, p.313-20, 2016.

SHANER, G.; FINNEY, R.E. The effects of nitrogen fertilization on the expression of slowmildwing in knox wheat. **Phytopathology**, St Paul, v.67, p.1051-5, 1977.

PASCHOLATI, S.F.; DALIO, R.J.D. Fisiologia do Parasitismo: como as plantas se defendem dos patógenos. *In*: AMORIM, L.; REZENDE, J.A.M.; BERGAMIM FILHO. A. (ed.) **Manual de fitopatologia**. Ouro Fino: Ceres, 2018, p.424-50.

PASSARDI. F.; COSIO, C.; PENEL, C.; DUNAND, C. Peroxidases have more functions than a Swiss army knife. **Plant Cell Reports**, Berlin, v.24, p.255-65, 2005.

PEEVER, T.L; OLSEN, L.; IBÁÑEZ, A.; TIMMER, L.W. Genetic diferentiation and host specificity among populations of Alternaria spp.causing brown spot of grapefruit and tangerine x grapefruit hybrids in Florida. **Phytopathology**, St Paul, v.90, p.407-14, 1999.

PETENÁ, G.; TANAKA, F.A.O.; MESQUITA, G.L.; BOARETTO, R.M.; ZAMBROSI, F.C.B.; QUAGGIO, J.A.; MATTOS-JR, D. Scanning electron microscopy of leaf and petal cuts of citrus trees fertigated with two nitrogen sources. **Citrus Research and Technology**, Cordeirópolis, v.37, p.218–25, 2016.

RESENDE, M.L.; COSTA, J.C.B.; CAVALCANTI, F.R.; RIBEIRO JÚNIOR, P.M.; CAMILO, F.R. Seleção de extratos vegetais para indução de resistência e ativação de respostas de defesa em cacaueiro contra Vassoura-de-bruxa. **Fitopatologia Brasileira**, Brasília. DF, v.32, p.213-21, 2004.

RENAUD, M.S.A.; AMORIM, L.; LOURENÇO, S.A.; SPOSITO, M.B. Escala diagramática para avaliação da mancha marrom de alternaria de citros. **Summa Phytopathologica**, Botucatu, v.34, n.3, p.270-1, 2008.

ROUT, J.R.; SAHOO, S.L. Antioxidant enzyme gene expression in response to copper stress in *Withania somnifera* L. **Plant Growth and Regulation**, Netherlands, v.71, p.95-9, 2013.

SILVEIRA, R.L.V.A.; HIGASHI, E.N.; CAMARGO, F.R.A.; SILVA, C.R. **Influência do estado nutricional do Eucalyptus na predisposição à ocorrência da ferrugem (*Puccinia psidii*)**. Piracicaba, 2003. 44 p. (Relatório Técnico)

STUART, R.M.; BASTIANEL, M.; AZEVEDO, F.A.; MACHADO, M.A. Alternaria brown spot. **Laranja**, Cordeirópolis, v.30, p.29-44, 2009.

TAIZ, L.; ZEIGER, E. **Plant physiology.** 3rd ed**.** Sunderland: Sinauer Associates, 2006. v.3, p.127-8.

TIMMER, L.W.; PEEVER, T.L.; SOLEIL, Z.; AZUYA, K.; KIMITSU, A. Alternaria diseases of citrus-novel pathosystems. **Phytopathologia Mediterranea**, Bologna, v.42, p.99-112, 2003.

YANG S.L.; CHUNG K.R. The nadph oxidase-mediated production of hydrogen peroxide (H_2O_2) and resistance to oxidative stress in the necrotrophic pathogen *Alternaria alternata* of citrus. **Molecular Plant Pathology**, Oxford, v.13, p.900-14, 2012.

YANG, S.L.; YU, P.L.; CHUNG, K.R. The glutathione peroxidase-mediated reactive oxygen species resistance, fungicide sensitivity and cell wall construction in the citrus fungal pathogen *Alternaria alternata*. **Environmental Microbiology**, Oxford, v.18, p.923-5, 2016.

WOUDENBERG, J.H.C.; SEIDL, M.F.; GROENEWALD, J.Z.; DE VRIES, M.; STIELOW, J.B.; THOMMA, B.P.H.J.; CROUS, P.W. Alternaria section *Alternaria*: species, formae speciales or pathotypes? **Studies in Mycology,** Baarn, v.82, p.1–21, 2015.