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Potassium Phosphite In Resistance Induction Of Green Mold In Post-Harvest Of "Ponkan" Tangerine

[1]Vinícius Franceschi, [1]Mycheli da Cruz, [2]Nean Dalacosta, [2]Janaina Bruzamarello, [2]Stheffani dos Santos, [1]Alberto Ricardo Stefeni, [3]Sergio Miguel Mazaro, [2]Elizabeth Koltz, [4]Milene Pereira

[1]Department of Agroecosystem, Federal TechnologicalUniversity of Paraná (UTFPR), Dois Vizinhos, PR, 85660-000, Brazil.
[2]Department of Agronomy, Federal Technological University of Paraná (UTFPR), Pato Branco, PR, 85503-390, Brazil.
[3]Department of Agronomy, Federal Technological University of Paraná (UTFPR), Dois Vizinhos, PR, 85660-000, Brazil.
[4]Department of Bioprocesses and Biotechnology, Federal Technological University of Paraná (UTFPR), Dois Vizinhos, PR 85660-000, Brazil.

Correspondence Author: Alberto Ricardo Janaina, Stefeni, Department of Agroecosystem, Federal Technological University of Paraná (UTFPR), Dois Vizinhos, PR, 85660-000, Brazil.
E-mail: albertostefeni@yahoo.com

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Abstract

Citriculture is an important activity in Brazilian agribusiness, due to the social, good and economic importance. However, the durability of these post-harvest fruits still on of the problems. The molds caused by *Penicillium digitatum* fungus, currently are among the main post-harvest diseases of fruits, affecting all species and citrus varieties. That disease is characterized by one soft rot on the fruit, which is covered with white mycelium with large style number, which confer a green color on affected local. This present work intended to evaluate different concentrations of potassium phosphite in the resistance induction of green mold in post-harvest of 'Ponkan'tangerine and the *P. digitatum* control *in vitro*. Fruits was picked at experimental orchard of Federal Technologic University of Parana – UTFPR – Dois Vizinhos – PR. After the collection, fruits and products was submitted to the treatment by immersion in different concentrations of phosphite. Afterwards, fruits were inoculated with spore suspension of *P. digitatum*. The design was completely randomized, with four replicates of 40 fruits, which were kept for 3 days at a temperature of 20 ° C (± 2), and then, performed as physical-chemical analyzes. The protein-related pathogenesis (PRPs) evaluation was determined by quantifying the activities of peroxidase, phenylalanine ammonia-lyase (PAL), chitinase and β-1,3-glucanase. The experiment *in vitro*, the phosphite was incorporated into the BDA medium (Potato-Dextrose and Agar) and then, the mycelial growth of *P. digitatum* was evaluated. Potassium phosphate doesn't interfere in the physico-chemical characteristics and at all the concentrations acted in resistance induction, activating enzymes PAL, peroxidase, and chitinase. The phosphites act in the control of P. digitatum in vitro, with fungicide action.

Key words: Rootings, penicillium digitatum, citrus

INTRODUCTION

The citrus culture present great food importance, whether on in natural consumption or for industry. In the international market, although treating citrus as the higher production like orange, the tangerines deserves special mention for their potential for in natural consumption (AZEVEDO, 2010). The molds caused by *Penicillium digitatum* fungus are among the main post-harvest diseases of fruits, affecting all species and varieties of citrus (LÓPEZ *et al*., 2015). That disease is characterized by one soft rot on the fruit, which is covered with white mycelium with large style number, which confer a green color on affected local. It causes significant losses to the production, since they compromise the physical and chemical quantity of the fruits (FISCHER *et al*., 2008).

Also, the principal control form of these disease is through the use of fungicides, however, the excessive use of chemical control can result in toxic residues in fruits and appearance *Penicillium strains* resistant of molecules fungicides (DANNER *et al*., 2008). Therefore, as an alternative to the excessive use of chemicals, as well as the search for products that are less aggressive to the environment and healthier from the point of view of the consumer, methods or products with potential for alternative control are studied (LADANIYA, 2008).

One product with potential in the integrated management of plant diseases is the phosphites. The phosphites (H2PO3) are leafs fertilizers formed by the reduction of a base of the phosphorous acid. Are rapidly absorbed by plants, as presents a high degree of solubility and mobility in the plant (via xylem and phloem), thus allowing various methods of application according to the type of plant and characteristics of the pathogen to be controlled (OGOSH *et al*, 2013).

Potassium phosphites have been used in several works in the search to confirm their mechanisms of action on the control of diseases, as on phytopathogens *in vitro* reducing mycelial growth and sporulation of fungi, *in vivo* can act in induction of resistance being capable of activating defense mechanisms, giving protection to the plants against microorganisms (MÜLLER, 2015;TÖFOLI *et al*.,2012; PEREIRA *et al*., 2012, SAUTTER *et al*., 2008).

In addition to the direct action on pathogens, studies have shown positive results in also post-harvest of apples, in studies by Spolti (2015), showed that potassium phosphite had fungicidal potential, with a 60% reduction in rot damage caused by *Cryptosporiopsis perennans*, and still inhibited the *in vitro* development of the fungus. According to McDonald *et al*. (2001), phosphite disrupts phosphorus metabolism in *Phytophthora* causing large accumulation of polyphosphate and pyriphosphate, their toxic effect is directly linked to their ability to interfere with fungal metabolism, increasing pyrophosphate and inhibiting the reaction of pyriphosphorylase. It also inhibits the activity of a series of enzymes of the glycolytic route and the oxidative pathway pentose phosphate (NEMESTOTHY & GUEST, 1990; JACKSON *et al*., 2000).

Also, the application of phosphite stimulates the production of biochemical responses in plant tissues against pathogens producing substances such as phytoalexins, which were produced by plants when it is infected by pathogens and are responsible for making them more resistant. Also, in induction of resistance

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occurs the accumulation of PR-proteins, such as peroxidases, chitinases and β-1,3-glucanase, also enzymes involved in the route of phenylpropanoids, such as phenylalanine ammonia-lyase, phytoalexins, among other pre and post-formed (STADNIK, 2000, PASCHOLATI; LEITE, 1995). Jackon *et al*., 2000, reports when the phosphite concentration within plant tissues is low, it reacts with the action of the pathogen at the point of penetration, stimulating the production of enzymes related to the plant defense. And when it is in high concentration, the phosphite acts directly on the pathogen inhibiting the growth of plants before it is able to establish an association with the host.

The direct action or fungicide of phosphites is the most reported, whereas the activator of defense mechanisms is scarce. In this sense, the present work intended to evaluate different concentrations of potassium phosphite in the resistance induction of green mold in post-harvest of 'Ponkan'tangerine and the *P. digitatum* control *in vitro*.

MATERIAL AND METHODS

The experiment was conducted at the Phytosanitary Laboratory of the Federal Technological University of Parana - Campus Dois Vizinhos, with Ponkan tangerine fruit from the UTFPR's orchard experimental field. The experimental design was the completely randomized with five treatments and four replicates, containing 40 fruits each one. The treatments consisted of different concentrations of phosphite (0,002, 0,004, 0,006 and 0,008%), and in the control was used distilled water. As source of potassium phosphite was used a commercial product Ultra K - Spraytec®.

Tests in vitro:

In the in vitro experiment, was evaluated the direct effect of the phosphite on the *P. digitatum* pathogen, for this purpose, the phosphite was filtered on the minipore®membrane and inserted into the PDA culture medium (Potato, Dextrose and Agar), sterile, melting yet. The culture medium was poured into Petri dishes, and in the center was inserted one disk of mycelium (5 millimetres – mm) from a pure *P. digitatum* colony. This pathogen was obtained by isolation of a Ponkan fruit, with green mold apparent symptoms, being minced in PDA medium until obtaining a pure colony. The concentrations used in the experiment were the same as in the post-harvest experiment. The Petri dishes were stored in BOD with controlled temperature (20ºC±2°C) and 12 hours of photoperiod.

Tests in vivo:

For in vivo experiment, the fruits were in contact by immersion in the phosphite solutions, to simulate a real situation of post-harvest treatment, being concerned about the agility of the process, subsequently two mechanical injuries (2 mm) were made in the equatorial region of each fruit and inoculated with 10 µL of *Penicillium digitatum* spore solution with a concentration of 1,103 spores.mL^{-1}. Afterwards the fruits were conditioned at a mean temperature of 20ºC (± 2) for three days, and that time was determined by daily evaluations to avoid the total rot of the fruits, when it was evaluated. The physical-chemical variables analyzed were: Rot incidence, diameter of lesions, total soluble solids (TSS), titratable acidity and loss in mass.

Induction of Disease Resistance:

At the biochemical analyzes were determined phenylalanine ammonia-lyase activity (PAL), peroxidase (POX), chitinase and β-1,3-glucanase. The rot incidence evaluation was performed visually and confirmed using a stereomicroscope. The lesion size was evaluated using a digital pachymeter, measuring the largest diameter of each lesion. The SST were measured from a manual refractometer and expressed in ºBx (degrees brix). The titratable acidity was determined in a 10 mL sample of fruit juice diluted to 100 mL of distilled water and titrated with sodium hydroxide solution 0.1 N up to pH 8.1 and expressed as grams (g) of citric acid per 100 mL (Equivalent gram = 64,02) (Association of Official Analytical Chemists - AOAC, 1997), the final result was obtained in percent (%). The mass in loss was obtained from the mass difference of the samples at the beginning (before the treatments) and the end of the experiment and expressed as grams.

For the biochemical analyzes, were removed plant tissue samples of 0,3g of each fruit at distance of 1 centimeter (cm) from the lesion and only from the treatment containing the highest dose (0,008%) compared to the control, as a result to be the only one to have effect on the reduction of lesion in the fruits. In total protein analyzes, the samples were macerated in a mortar with 4 mL phosphate buffer 0,2 M (pH 7,5). Subsequently, the material was centrifuged (14.000g for 10 min at 4°C) and the supernatant collected. To quantify the total protein content of the samples, was used the Bradford (1976) test. The total protein reading was performed in a spectrophotometer at 590 nanometers (nm), using bovine serum albumin as standard.

Phenylalanine ammonia-lyase activity (PAL) was determined by colorimetric quantification of trans-cinnamic acid released from the phenylalanine substrate, according to the methodology described by Kuhn (2007), where it is indicated to use 0,3 g of the sample with 3,0 ml of the TRIS-HCl buffer pH 8,0. This extract was packaged in suspended tubes and centrifuged for 10 minutes at 4°C to 6000 rpm. After, 200 microliter aliquot (µL) was transferred to the test tube, adding another 3,0 mL of the extraction buffer. The solution was shaken in the vortex machine, thus obtaining the enzyme extract. From this extract, was transferred 1,5 mL to another test tube, with 1,0 mL of the extraction buffer more and 0,5 mL of phenylalanine. Again, the solution was churned in vortex for homogenization. Upon, the tubes were incubated in water-bath for 45 minutes at 40ºC. Afterwards, the tubes were incubated in a water bath for 45 minutes at 40°C. After being removed from the water bath, the tubes were placed in an ice bath for 5 minutes to stop the reaction an than can be realized reading in spectrophotometer at 290 nm, the results had been made Uabs. mg^{-1} of protein.

The peroxidases were determined by the method recommended by Matsuno; Uritani (1972), The samples were macerated with 4,0 mL 0,05 M phosphate buffer (pH 7) plus 0,005 g polyvinylpyrrolidone. The extract was packaged at Eppendorf tubes, centrifuged for 20 minutes at 4°C and 5000 rpm. After centrifugation, 2,0 mL of the supernatant was withdrawn and placed in test tubes containing 3,0 mL citrate buffer (pH 5.0) plus 0,5 mL 3% hydrogen peroxide and 0,5 mL guaiacol, 0,5%. Also, was added 0,5 mL of sodium bisulfite and the reading the reading carried out at 450 nm in spectrophotometer, the results were expressed as absorbance unit per minute in milligrams of proteins (Uabs.min^{-1}.mg protein^{-1}).

To determine the activities of chitinases and β-1,3-glucanase were followed the procedures described by Wirth; Wolf (1992), with ad equations, and the samples were macerated in 4,0 mL of 100 Mm acetate buffer (pH 5.0), with subsequent centrifugation (20.000 g for 25 min at -4°C). The supernatant was collected and used for the enzyme activity evaluation. The enzymatic activity of chitinase was evaluated by the release of soluble oligomers "chitin-azure", from carboxymethyl chitin marked with bright remazol violet 5R -RBV (Sigma Aldrich®). For the spectrophotometric determination of the β-1,3-glucanase activities in the extracts, the bright curdlan-remazol blue substrate (Sigma Aldrich® - 4 mg.ml^{-1}) was used as the substrate, the results were expressed as enzymatic units per milligram of protein (U.E.mg^{-1} protein).

Statistical Analysis:

The data were submitted to analysis of variance by the F test (α0.05) and the differences between the means of the treatments, compared by the Tukey test (α0.05) through the statistical analysis program Assistat Software (SILVA &AZEVEDO, 2009).

RESULTS AND DISCUSSION

Change in inhibited the growth of fungus:

Potassium phosphites have shown potential in the control of phytopathogens, due to their fungicidal and fungistatic effect, which confirmed in the results obtained in the in vitro experiments, all the concentrations of potassium phosphite tested had a fungicidal effect on P. digitatum, thus confirming a direct effect on the pathogen. In vitro tests, have shown that phosphites cause changes in the growth and reproduction of phytopathogens. The fungitoxicity of the phosphites varies between different species and depends mainly on the exposure of the pathogen to these compounds. In general, the protection conferred by phosphites can interfere with fungus metabolism and trigger a fungicidal effect, as well as reduce mycelial growth and fungal sporulation and develop a fungistatic effect (CARMONA, 2011).

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For post-harvest, the results showed that all fruits presented characteristic green mold rot, indicating the efficiency of the inoculation process, as well as the aggressiveness of the P. digitatum pathogen. The application of potassium phosphite had an effect on the reduction of lesion size in the fruits, with a linear effect according to the increase of the concentrations (Figure 1). Thus, at the highest concentration (0.008%), it obtained a reduction of 11.6% in relation to the control.

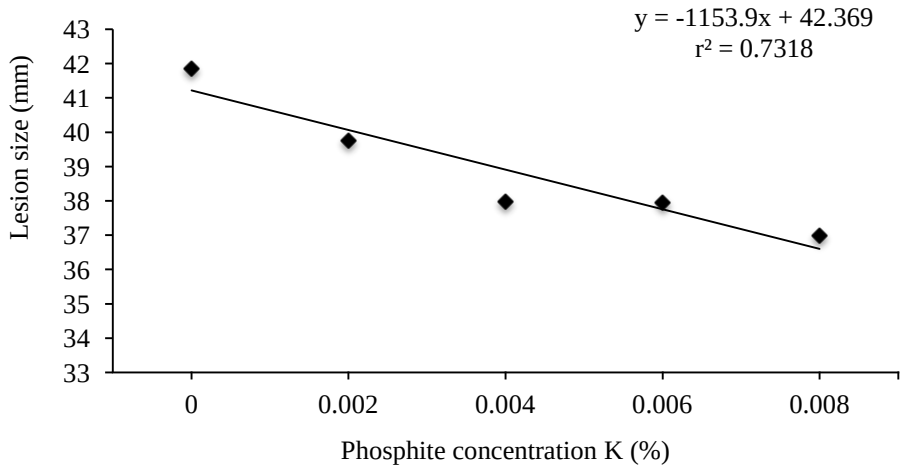

Fig. 1: Effect of phosphite concentrations of K on the diameter of lesions caused by *P. Digitatum* on Ponkan tangerine fruits. Dois Vizinhos-PR, 2017.

The effects of the phosphites confirmed *in vitro*, in the field can result in a reduction in the incidence and severity of disease. As well as occurred in the present work, where the reduction of the lesions size caused by green mold on Ponkan tangerines, confirms the performance of the phosphites in the diseases control, being it possible for their fungistatic potential, acting directly on the development of the pathogen, or resistance induction, acting in enzymatic processes and in the preservation and integrity of the fruits, delaying the colonization process of the fungus.

In work with *Pythium sp.* e *Fusarium semitectum*, phosphite of K demonstrated fungitoxic action, since that completely prevent the fungus development (CAIXETA *et al*,2012). In studies by Daniel and Guest (2006) the phosphites failed to paralyze the mycelial growth of Oomycetes, but rather contain them. Also, studies demonstrate the potential of phosphites in other patosystems such as mildew, powdery mildew, gala leaf spot (*Colletotrichum gloeosporioides*) and apple scabs (*Venturia inaequalis*). Being effective in the management of oomycetes like *Phytium spp.* and *Phytophthora spp.* and fungus causing collar rot, root, trunk and fruit (WICKS *et al*., 1990, MCDONALD *et al*., 2001, BRACKMANN *et al*., 2004), as well as in the control of blue mold on apples caused by *Penicillium expansum* (BLUM *et al*., 2007).

Change in Fruit Parameters:

No significant differences were observed with the use of different concentrations for the variables of loss in mass, TSS and titratable acidity, thus indicating that the phosphite doesn't interfere in the normal process of fruit maturation (Table 1). Stella *et al*. (2013) also observed absence of changes in firmness and texture attributes in 'Fuji' apples treated in pre-harvest with K phosphite, with fruit quality maintenance after harvest.

Table 1: Mass in loss (g), titratable acidity (%) and total soluble solids (^{o}Bx) in postharvest of "Ponkan" tangerines. DoisVizinhos - PR, 2017.

Treatments	Variables		
	Mass (g)	Acidity (%)	TSS (^{o}Bx)
Control	0.030 $*^{ns}$	0.57 $*^{ns}$	9.30 $*^{ns}$
0.008%	0.036	0.57	9.50
0.006%	0.035	0.60	8.67
0.004%	0.035	0.60	9.30
0.002%	0.035	0.55	8.82

NS= Averages not significant by Tukey's test (α CV (%): coefficient of variation

Changes in Some Metabolic Activities:

Plants treated with phosphorous acid and after inoculation of the pathogen, showed defense-related changes, such as hypersensitivity, core migration and phytoalexin accumulation around the challenged cells (DANIEL *et al*., 2005). Mechanisms involved in the resistance induction. The effect of phosphite on enzymatic activity on Ponkan fruits can be observed by increasing the activity of PAL, peroxidase and chitinase in comparison with the control (Figure 2), demonstrating the activation of defense responses. No β-1,3-glucanase activity was observed.

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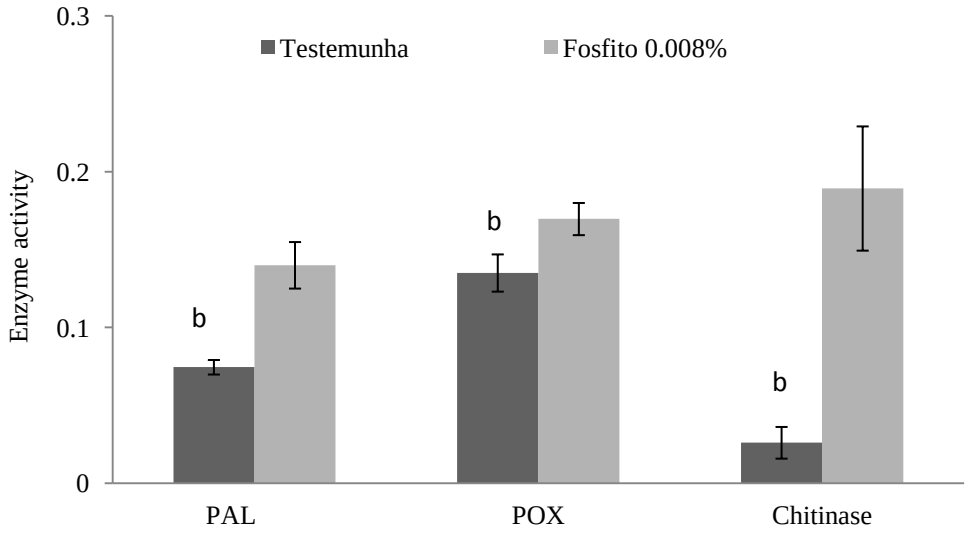

Fig. 2: Determination of PAL (Uabs mg -1 protein), Peroxidase (Uabs.min-1.mg protein-1), Chitinase (UEmg -1 protein) as a function of the application of Potassium Phosphite (K) in the concentration 0.008% in relation to the control. Averages differ from one another by the Student test (p> 0.05). Bars indicate standard error. Dois Vizinhos - PR, 2017.

It was observed that potassium phosphites acted on induction of resistance by activating enzyme PAL, peroxidase and chitinase. It can be said that potassium phosphite activated the route of the phenylpropanoids, in other words, mechanisms that involve the synthesis of lignin and phenolic compounds were potentiated, this route is confirmed by the activation of the two main enzymes involved in its expression, PAL and POX.

The activation of the PAL, indicates an activation of the secondary metabolism, since it is responsible for the deamination of L-phenylalanine, transforming into trans-synamic acid, integrating several phenolic compounds like esters, coumarins, flavonoids and lignins(SCHWAN -ESTRADA *et al*., 2008). This enzyme can also be activated by environmental factors such as low levels of nutrients, light and fungal infection. The fungal invasion can trigger the transcription of the messenger RNA encoding the PAL, increasing the amount of this enzyme in the plant, stimulating the synthesis of phenolic compounds (TAIZ & ZEIGER, 2013).

The POX participate in the lignification process of the plant cells, being able to affect fungal development, through physical barriers and also reducing the nutrients diffusion, compromising colonization of the fungus, or even synthesizing toxic lignin precursors (PASCHOLATI & LEITE, 1995). At the highest phosphites concentrations, the performance of these enzymes coincides with the lower incidence of diseases in the fruits.

Was evaluates the activity of chitinase and β-1,3-glucanase, where there was only chitinase expression, a fact that may be related to the characteristics of the pathogen, since the fungus *Penicillium digitatum* belongs to the *Ascomycota* phylum, and its cell wall is constituted of chitin. Filamentous fungi have chitin as the main component of the cell wall and produce chitinases at all stages of their development, from spore germination to mycelial growth (GOODAY, 1990).

It is believed that there was specificity in the expression of the enzyme chitinase, for plant defense. The role of chitinase in pathogen interactions with plants is to efficiently hydrolyze chitin, as already mentioned, the main cell wall component of many fungi (SCHWAN-ESTRADA *et al*., 2008).

Conclusion:

Potassium phosphite exerts a fungicidal function on the *P. digitatum* pathogen under *in vitro* conditions. Potassium phosphite applied in post-harvest, reduces the lesions size in Ponkan tangerine fruits inoculated with *P. digitatum* and activates the plant defense enzymes PAL, POX, and chitinase.

Potasium phosphate applied in post-harvest of Ponkan tangerine doesn't interfere in the physico-chemical characteristics of loss in mass, TSS and titratable acidity of fruits.

Future Studies:

In future works, it's suggested to identify the inhibitory effect of the compounds present in the phosphites and test the application in pre-harvest, because it's believed the product when applied in pre-harvest can contribute to the fruits production more resistant to molds and rotting. As well as studies on the application of compounds under controlled conditions evaluating their effect on the germination and fungus infection.

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