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Control of Phytophthora brown rot of lemons by pre- and postharvest applications of potassium phosphite

A. C. Ramallo · L. Cerioni · G. M. Olmedo · S. I. Volentini · J. Ramallo · V. A. Rapisarda

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Abstract Citrus Brown Rot (BR), caused by *Phytophthora* spp., provokes important economical losses mainly in periods of high rainfall. The management of this disease in Florida and Brazilian citrus areas, main orange growers worldwide, includes chemical control using phosphite salts. In Argentina, the world leader in lemon production, these compounds are registered only as fertilizers. In this work, the effect of potassium phosphite on different *Phytophthora* sp. cellular structures and the conditions to control lemon BR by it application at pre and post-harvest stages were evaluated. Phosphite inhibited in vitro the mycelial growth, the sporangia production, and the motility and germination of zoospores of a local isolate of *Phytophthora citrophthora*. In postharvest applications on artificially inoculated lemons, the phosphites exerted a moderate curative activity, reducing BR incidences ~25% in respect to controls. When this salt was applied a week before inoculation, BR incidences were 50–60% lower than those of controls, denoting a significant preventive activity. The application of phosphite with fungicides in commercial packingline prevented BR disease in fruit inoculated at 96 h post-treatment. In pre-harvest, two phosphite applications reduced incidences ~40–60% in lemons harvested and inoculated up to 75 d after treatment. Our data confer valuable technical information towards the use of phosphite salts against lemon BR, contributing to the pre- and postharvest management strategies of this disease.

Keywords Citrus · Brown rot · *Phytophthora citrophthora* · Potassium phosphite

A. C. Ramallo · L. Cerioni · G. M. Olmedo · S. I. Volentini · V. A. Rapisarda (✉)
Instituto Superior de Investigaciones Biológicas (INSIBIO), CONICET-UNT, and Instituto de Química Biológica "Dr Bernabé Bloj", Facultad de Bioquímica, Química y Farmacia, UNT. Chacabuco 461, T4000ILI San Miguel de Tucumán, Argentina
e-mail: vrapisarda@fbqf.unt.edu.ar

J. Ramallo
Laboratorio de Desarrollo e Investigación, SA San Miguel, Lavalle 4001, T4000BAB San Miguel de Tucumán, Argentina

Introduction

Brown rot (BR) is a disease of citrus fruit caused by *Phytophthora* spp. (Oomycota: Stramenopila) that leads to important economic losses worldwide, with a high incidence in wet growing areas (Eckert and Brown 2000; Adaskaveg and Förster 2014; Graham and Dewdney 2014; Graham and Feichtenberger 2015). BR decayed lemons exhibit an olive-brown discoloration of the rind and a characteristic rancid odor. Frequently, asymptomatic infected fruit is harvested and packed, and the symptoms appear during storage (Graham and Timmer 2003). This affects the production per se and favors fruit colonization by other postharvest wound pathogens, such as *Penicillium* spp. and *Geotrichum* spp. (Adaskaveg et al. 2015).

Several species of *Phytophthora* are associated with the disease, being *P. citrophthora* (Sm. & Sm.) Leonian and *P. nicotianae* Breda de Haan (synonymous with *P. parasitica* Dastur) the most widespread and important

species (Erwin and Ribeiro 1996; Feichtenberger 2001; Graham and Menge 2000). The relative importance of the species varies with time of year, being *P. nicotianae* more active in warmer seasons, whereas *P. citropthora* occurs throughout the year (Adaskaveg and Förster 2014). *Phytophthora* spp. persist in the soil as oospores, chlamydospores or as mycelium associated with decayed fruit material. The disease cycle begins with the production of sporangia that are developed from all these cellular structures when the soil moisture content is high. Sporangia release a large number of mobile zoospores, which may encyst and germinate to form mycelia under adequate environmental conditions of wetness and temperature (Eckert and Eaks 1989; Adaskaveg and Förster 2014). Rain and wind favor the infection of fruit and young foliage by zoospores splashed from the soil (primary infections) or from the above-ground parts of the plant (secondary infections).

BR management is based on pre-harvest cultural practices such as removing lower tree branches, avoiding harvest of low-hanging fruit, applying a proper irrigation and chemical control (Eckert and Brown 2000). In the United States, BR chemical control is currently performed using phosphonates (e.g. fosetyl-Al) and phenylamide (e.g. metalaxyl) before harvest (Adaskaveg et al. 2015). Additionally, phosphite salt sprays are widely used in Florida and Brazilian citrus areas, the main orange production areas of the world (Graham and Feichtenberger 2015; USDA 2018). Graham (2011) reviewed the role of inorganic salts of phosphorous acid in the control of Phytophthora diseases in citrus and indicated that they are highly systemic in the tree. Phosphite salts present a complex mode of action against *Phytophthora* spp. involving direct inhibition and indirect response (Fenn and Coffey 1984; Graham 2011; Smillie et al. 1989; Guest and Grant 1991; Daniel and Guest 2006). Evidence suggests that phosphites induce a phosphate starvation in the pathogen, causing the release of stress metabolites that elicit a vigorous defense response in the host plant (Perez et al. 1995; Smith et al. 1997; McDonald et al. 2001), including the synthesis of structural polyphenolics and phytoalexins (Dixon et al. 2002).

Even though many nutritional and fungicidal phosphite products are marketed throughout the world (Leymonie 2007; Thao and Yamakawa 2009), phosphite salts are only registered as fertilizers in Argentina (SENASA 2009). Argentina produced more than 20% of the world's lemons crop during 2016 (Federcitrus 2017). 80% of this production is located in Tucumán province, between 26 and 28°S latitude, where environmental conditions favor the development of BR (Federcitrus 2017; Palacios 2005). This production area is 10,000–17,000 km from the overseas markets in Europe, Asia and USA, thus fresh fruit require 25–40 days to get to the final consumers. The management of BR represents a constant challenge for local producers, since scarce technical information about the disease and the control strategies is available. At present, BR management is performed with pre-harvest applications of copper salts, without an efficient control of the disease. The aims of this work were to evaluate in vitro the effect of potassium phosphite on different *Phytophthora* sp. cellular structures and to establish the conditions to control lemon BR in our region by its application at pre and post-harvest stages.

Materials and methods

Chemical sources

Four commercial trademarked formulations of potassium phosphite were used: PhiA (45.5% a.i., potassium phosphite AFITAL, AgroEMCODI, Argentina), PhiB (39.0% a.i. potassium phosphite BioBloemen, Química Bloemendaal S.R.L., Argentina), PhiF (55.5% a.i. potassium phosphite Fighter, Africhem S.A., South Africa) and PhiP (52.0% a.i. potassium phosphite Phit, Siner S.A., Argentina). Conventional postharvest fungicides thiabendozale (TBZ; 42.9% a.i., Tecto 500SC, Syngenta) and pyrimethanil (PYR; 30.0% a.i., Mythos, Bayer Crop Science) were also employed.

Pathogen isolation, DNA extraction and molecular identification

Phytophthora sp. was isolated from naturally decayed lemons collected from soil surface of commercial orchards in Tucumán (Argentina). Pieces of affected albedo were placed onto water agar (WA) plates amended with rifampicin, ampicillin and nistatin, according to Jeffers and Martin (1986), and incubated at 24 °C for 7–10 d. Then, individual WA disks with mycelia were placed onto V-8 juice agar plates (Mitchell et al. 1986) and incubated at 24 °C for 7–10 d. Phenotypic identification of the *Phytophthora* isolate was based on the taxonomic keys described in *Practical Guide to*

Detection and Identification of Phytophthora (Drenth and Sendall 2001). For DNA extraction and pathogen molecular characterization, protocols described by Möller et al. (1992) and White et al. (1990) were followed. Briefly, DNA was extracted from the fungal culture and primers ITS4 and ITS5 were used to amplify the internal transcribed spacer ITS1 and ITS2, including the 5.8 S region of rDNA. Species identification was confirmed according to Martin et al. (2004) using FMPh-8b and FMPh-10b primers which amplified the conserved sequences corresponding to the 3′ end of coxII gene, the 5′ end of coxI gene and the spacer region between them. All PCR products were purified and sequenced at CERELA-CONICET, Tucumán-Argentina. Sequences were edited using the program Sequencing Analysis 5.3.1 and aligned using BLAST of the National Center for Biotechnology Information, National Institutes of Health USA (Bethesda, MD).

Mycelial growth, sporangia production and zoospores motility and germination

Individual V-8 agar disks (5 mm in diameter) of *Phytophthora* sp. mycelium were obtained from the edge of an active growing colony and placed onto potato dextrose agar (PDA) plates amended with potassium phosphite (PhiA), PYR or TBZ at final concentrations of 1, 10, 100 and 1000 mg a.i./l. Plates containing only PDA were used as controls. After a 7-d incubation period at 24 °C in darkness, the radial growth of mycelia was measured. The chemical's effective concentrations causing 50 or 90% of mycelial growth reduction (EC50 or EC90, respectively) were calculated from the fitted regression line of the probit-transformed percent inhibition plotted against the log-transformed fungicide concentration (Brantner and Windels 1998; King-Watson 1988). To evaluate sporangia production in the presence of phosphite, mycelium disks were placed in V8 broth medium for 24 h. Then, medium was removed and replaced by distilled water amended with potassium phosphite (PhiA) to give final concentrations of 10, 100, or 1000 mg a.i./l. After 2 d of incubation at 24 °C in continuous light, sporangia formation was evaluated by direct observation with an inverted light microscope Olympus IX51 equipped with an Olympus digital camera. Zoospores release was induced by incubating sporangia in water at 4 °C for 20 min. Suspensions (2.5×10^4 zoospores/ml) were prepared and treated with potassium phosphite at final concentrations of 1, 10, 100, and 1000 mg a.i./l. Zoospore suspensions were observed microscopically to determine the percentage of mobile cells at each incubation time, considering only translational motion (motion by which a zoospore shifts from one point in space to another). Zoospore germination was evaluated after 24 h of incubation at 24 °C in the dark, by counting germinated cells from the total. In all cases, controls were performed without potassium phosphite.

Fruit inoculation

Eureka lemons were collected from commercial orchards and stored at 7 °C and 90% RH until tests were performed. Fruit were used without any postharvest treatment or coating. For inoculation, the local *Phytophthora* sp. isolate was grown on V8 agar plates at 24 °C for 7 d in darkness, and then exposed to continuous light for 3–4 d. Mycelial plugs (4 mm in diameter) were cut aseptically from the growing edge of colonies and placed on the stylar end surface of each lemon without wounding. Fruit were stored in plastic trays at 24 °C and 90% RH. After 24 h, mycelial plugs were removed from lemon surfaces. BR incidence on lemons was determined after further 6 d of incubation.

Laboratory treatments with potassium phosphite on postharvest lemons

Potassium phosphite treatments were performed after fruit inoculation (curative activity) or before inoculation (preventive activity). For both evaluations, lemons were immersed for 15 s in aqueous solutions of PhiA, PhiB, PhiF and PhiP at 2000 mg a.i./l. Controls consisted of lemons treated with water. In preventive assays, lemons were inoculated immediately after treatments or after 7 d of storage at 24 °C and 90% RH. BR incidence was recorded as described above.

Commercial packing-line treatments with potassium phosphite and fungicides on postharvest lemons

Lemons were treated in a commercial packing-line by aqueous spray application over rotating natural bristle brushes at 85–90 rpm. Fruit were treated with a blend of 2000 mg a.i./l potassium phosphite +3000 mg a.i./l TBZ + 1000 mg a.i./l PYR. Assayed fruit were recovered from the packingline and inoculated after 0, 24, 48, 72 and 96 h, as previously described. Controls consisted

of lemons treated with water. BR incidence was recorded as stated above.

Pre-harvest treatments with potassium phosphite on lemon trees

Experimental plots were established in commercial orchards of lemon trees Lisboa Limoneira 8A/Flying Dragon from Santa Mónica, Famaillá (Tucumán, Argentina) in 2016 and 2017 production seasons. The groves were maintained following conventional commercial practices without the application of fungicides. The experimental design consisted in 4 randomly distributed blocks of 10 trees each, including 5 consecutive trees treated with potassium phosphite (PhiP) and 5 control trees sprayed with water. PhiP was applied on trees by a high-pressure handgun (80 psi), spraying to run-off with ~ 8 l/tree. In 2016, lemon trees were exposed to a single application on February 12th (austral summer season). In 2017, trees were treated twice, on February 4th and 24th. Fruit were harvested 30, 60, 75 and 100 d after the last phosphite application, brought to the laboratory and inoculated. BR evaluation was carried out as previously described.

Statistical analysis

For in vitro assays, three replicates of each condition were performed and assays were repeated three times. In postharvest trials, each treatment was applied to 4 replicates of 15 fruit and assays were conducted twice. In pre-harvest trials, four replicates of 20 lemons were arbitrarily collected at each harvest time and the whole experiment was conducted twice. Data were analyzed by using an ANOVA analysis followed by Fisher's protected least significant difference calculated at $P = 0.05$ (SPSS Statistics 17.0).

Results

Phytophthora citrophthora inhibition by potassium phosphite

A local phytopathogenic isolate obtained from infected fruit was phenotypically identified as *Phytophthora* sp. In the molecular characterization, the sequence amplified with ITS primers (deposited in Genebank, accession number MK069991) presented a 98–99% identity with four different *Phytophophtora* sp.: *Phytophthora meadii* (KY212025), *P. botryosa* (JN618705), *P. citropthora* (KY608277), and *P. colocasiae* (JN661147). Since the ITS region is insufficient for differentiating closely related species, the conserved mitochondrial sequence corresponding to the 3′ end of *cox*II gene, the 5′ end of *cox*I gene and the spacer region between them was further used (Martin et al. 2004; Grünwald et al. 2011). The obtained citochrome oxidase sequence, deposited in Genebank as MK073923, exhibited 98% identity only with the reference sequence KY608283 that correspond to *P. citropthora*; hence our isolate was identified as *P. citropthora*.

Potassium phosphite inhibited mycelial growth of *P. citrophthora* with an EC50 value of 76.15 mg a.i./l ± 10.5 and an EC90 of 701.5 mg a.i./l ± 89.5. This salt also inhibited sporangium production at 1000 mg a.i./l (Fig. 1a) in a concentration dependent manner. A reduction of 65.5% of zoospore germination was achieved with 1000 mg a.i./l in respect to water control (Fig. 1b). Moreover, zoospore motility was impaired in all the phosphite concentrations evaluated (Fig. 1c). Only 20% of zoospores were moving at 100 min of incubation with 1 mg a.i./l, while 80% were still moving in the water control. The period of time required to stop the movement at 1 and 10 mg a.i./l were 250 and 175 min, respectively, whereas at 100 and 1000 mg a.i./l the zoospore movement ceased by 50 min.

Curative and preventive actions of potassium phosphite against lemon BR in laboratory trials

Curative treatments with the four phosphite formulations moderately controlled lemon BR, reducing incidences by 20–40% when compared to water treated fruit (Fig. 2). Laboratory preventive treatments showed that there was limited control of BR in lemons inoculated immediately after phosphite applications (Fig. 3). The salt effectiveness increased when fruit was inoculated 7 d after treatments. In such conditions, all the tested formulations reduced disease incidence around 50% in respect to the water control.

Preventive action against lemon BR of commercial application of potassium phosphite and fungicides

The BR relative incidence after preventive treatment with a mix of PhiA+PYR + TBZ applied on a commercial packingline is shown in Fig. 4. When inoculation

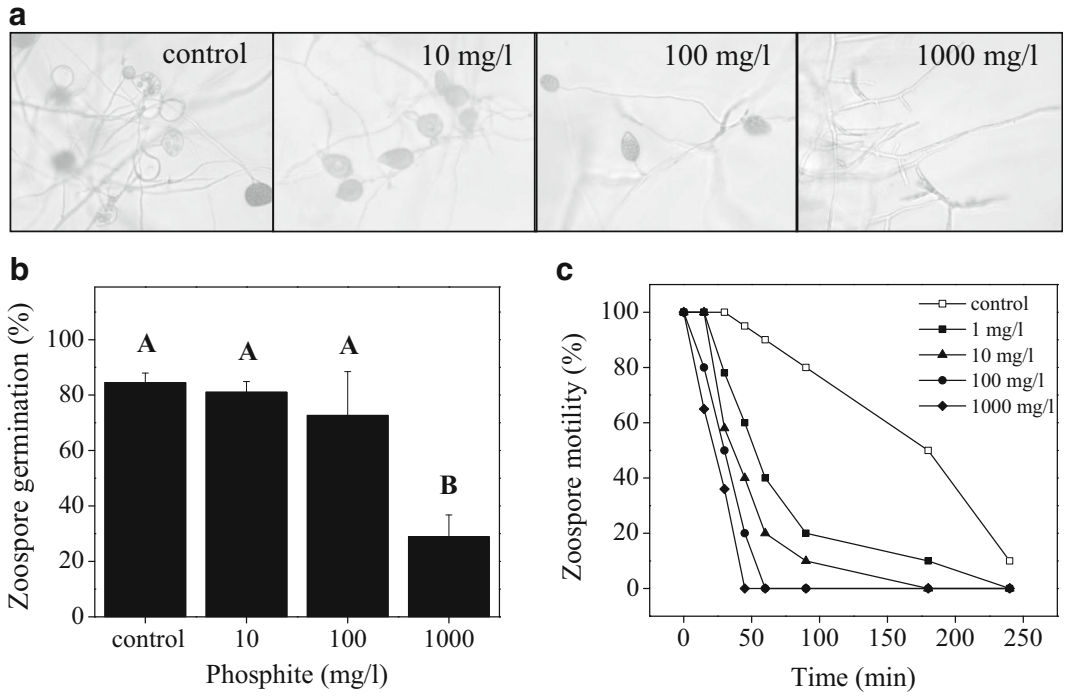

Fig. 1 In vitro effect of potassium phosphite on *Phytophthora citrophthora*. Sporangia formation (**a**), zoospore germination (**b**) and zoospore motility (**c**) in the presence of different potassium phosphite concentrations. In panel **b**, different letters indicate significant differences according to Fisher's protected LSD with a p value ≤0.05

was performed immediately or up to 72 h after treatment, the control of the disease was null or poor. Interestingly, the incidence was reduced by around 40% when lemons were inoculated at 96 h after treatment. It is worth to note that, in laboratory trials, only 15–20% control of BR was achieved when lemons were immersed in 3000 mg a.i./l TBZ or 1000 mg a.i./l PYR for 15 s and immediately inoculated (data not shown).

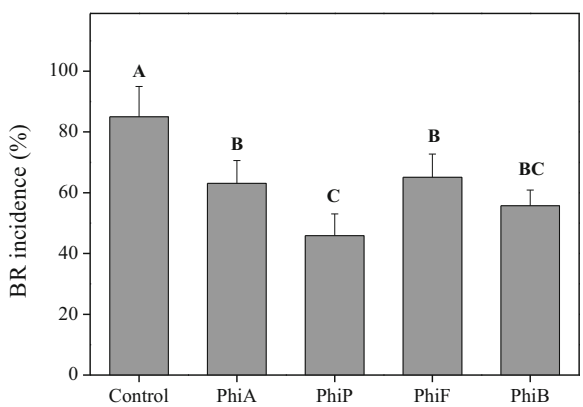

Fig. 2 Curative action of different potassium phosphite formulations against Brown Rot. Lemons were artificially inoculated with *P. citrophthora* and immersed in 2000 mg/l potassium phosphite solutions for 15 s. Different potassium phosphite formulations were employed: PhiA (AFITAL), PhiB (BioBloemen), PhiF (Fighter), and PhiP (Phit). Different letters indicate significant differences according to Fisher's protected LSD with a p value ≤0.05

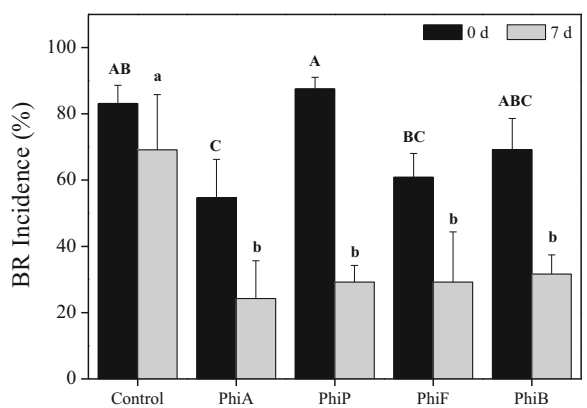

Fig. 3 Preventive action of different potassium phosphite formulations against Brown Rot. Lemons were immersed in potassium phosphite solutions at 2000 mg/l for 15 s, and inoculated immediately or after 7 d of storage. Different letters indicate significant differences among lemons inoculated at 0 d (capital letters) or 7 d (small letters) according to Fisher's protected LSD with a p value ≤0.05

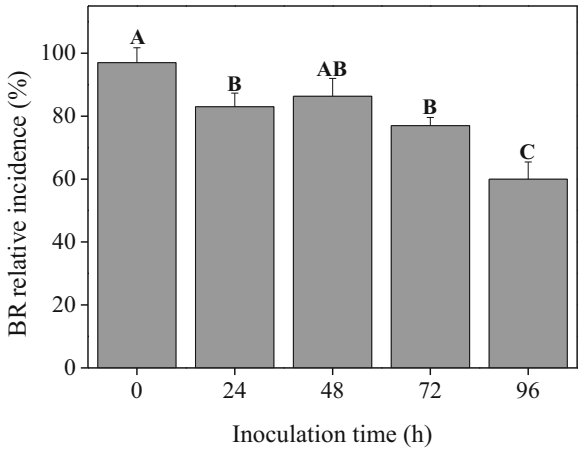

Fig. 4 Preventive action of commercial packingline application of potassium phosphite with fungicides against Brown Rot. Lemons were treated on a commercial packingline with a combination of 2000 mg/l potassium phosphite, 3000 mg/l TBZ, and 1000 mg/l PYR. Fruit were recovered at 24, 48, 72 and 96 h and inoculated with *P. citrophthora*. Values were expressed as BR relative incidence (%) in respect to the corresponding controls. Different letters indicate significant differences according to Fisher's protected LSD with a p value ≤0.05

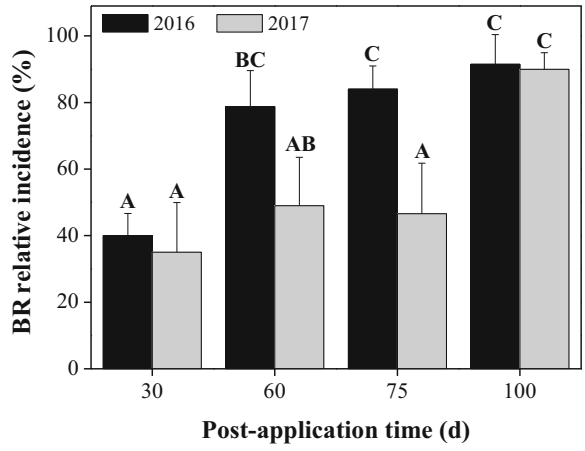

Fig. 5 Preventive action of preharvest applications of potassium phosphite against Brown Rot on postharvest lemons. Trees were sprayed once (2016) or twice (2017) with 2000 mg/l potassium phosphite. Lemons were harvested at the indicated times post-applications and immediately inoculated with *P. citrophthora*. Values were expressed as BR relative incidence (%) in respect to the corresponding controls. Different letters indicate significant differences according to Fisher's protected LSD with a p value ≤0.05

Moreover, the *P. citrophthora* isolate was insensitive to TBZ (EC50 > 2000 mg a.i./l), while it had low sensitivity to PYR (EC50 and EC90 values of 11.8 and 67.5 mg a.i./l, respectively).

Preventive action against lemon BR of pre-harvest application of potassium phosphite

Figure 5 shows BR relative incidence in lemons harvested from trees previously treated with phosphite and artificially inoculated. With a single application (2016 season), the relative BR incidence was around 40% in lemons harvested 30 d post-treatment. When harvest and inoculation were performed from 60 to 100 d post-treatment, the disease control was lower with incidences >80%. On the other hand, when trees were sprayed twice (2017 season), phosphite preventive action against BR was evident until 75 d post-treatments, having incidence values between 40 and 60%.

Discussion

Potassium phosphite was tested in vitro against a local isolate of *P. citrophthora* obtained from naturally infected lemon fruit. Phosphite was able to inhibit different growth stages of the pathogen, severely affecting sporangia production and zoospore germination. The direct inhibitory action of this salt on mycelial growth of other *Phytophthora* spp. has been previously reported by Smillie et al. (1989) and Adaskaveg and Förster (2014), with EC90 values similar to the present results. Nevertheless, our studies represent the first report on phosphite effects on other stages of the pathogen growth.

Curative action of phosphite salts on the pathosystem *P. citrophthora* - *Citrus limon* was moderately efficient (20–40% incidence reduction) at 2000 mg a.i./l after 24 h of fruit inoculation. As reported Adaskaveg et al. (2015), the application of potassium phosphite at 1500 mg/l on Valencia oranges inoculated 18 h before resulted in approximately 96% BR control. This discrepancy indicates that the efficiency of phosphite salts to control BR disease in curative treatments should not be generalized. It should be considered the differences in the citrus cultivar used (lemons instead of oranges), the inoculation method (mycelia plugs instead of zoospore suspensions), and the attributes of the pathogenic isolates. Although the products were provided by different trademarks, formulations used in both studies contain the same active principle (potassium phosphite); thus, it is not attributed the observed dissimilar result to this topic.

Based on our results, the preventive effect of phosphite salts was more marked than the curative action. Data suggests that some response might be triggered on fruit by the treatment, as the preventive BR control by potassium phosphite was evident in lemons treated 7 d before inoculation. Although not evaluated in this work, the responses could be chemical and/or anatomical, e.g. synthesis of protective substances, changes in cell wall that could produce mechanical barriers, among others. Further investigation of this topic is needed.

Phytophthora spp. could spread from infected to healthy fruit during several postharvest stages like de-greening, storage or transit (Adaskaveg et al. 2015). In this work, when the combination of postharvest fungicides (TBZ and PYR) and potassium phosphite was applied on lemons in the commercial packing-line, BR was controlled on fruits inoculated 96 h after treatment. The result is important as, at the beginning of the harvest season in Tucumán-Argentina, treated fruit is usually stored during 96 h in the de-greening chamber. In line with the preventive laboratory assays, time seems to be crucial to develop lemon responses against the infection. Together, phosphite salt application in packinghouses could contribute to prevent post-harvest dissemination of BR. Moreover, this salt controlled other important lemon diseases, such as green and blue molds, sour rot and phomopsis stem-end rot (Cerioni et al. 2013a, b).

A single pre-harvest application of potassium phosphite on lemon trees provided protection against *P. citrophthora* in fruit harvested 30 d post-treatment. Graham (2011) previously reported that a foliar application of potassium phosphite on Hamlin oranges provided 60 to 90 d of disease control in fruit inoculated with *P. palmivora*. In our assays, the time lapse of fruit protection was prolonged up to 75 d post-treatment when trees were sprayed twice. The single application used by Florida and Brazilian citrus growers (Graham and Feichtenberger 2015) seems to be insufficient for the lemon production in our region. Even though more study is needed to understand the cause of this phenomenon, the improving results is promising as a strategy for the control of lemon BR.

Taken together, preventive action by potassium phosphite against lemon BR is evident in both, pre- and postharvest stages. It is recommended a double phosphite application on groves several days before harvest time and the combination of phosphite salts with fungicides in postharvest treatment of lemons. Our data give valuable technical information that contributes to the management strategies of BR and represent a suitable tool for the disease control in Argentinean lemon production.

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