Asian Journal of Agricultural and Horticultural Research

Volume 10, Issue 4, Page 496-506, 2023; Article no.AJAHR.106207
ISSN: 2581-4478

Exploring Alternative Products for Tomato *Septoria lycopersici* Control

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJAHR/2023/v10i4289

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/106207

Original Research Article

Received: 13/07/2023
Accepted: 20/09/2023
Published: 14/11/2023

ABSTRACT

The septoriose (*Septoria lycopersici*) is an important disease in tomato production and can lead to significant losses. Although there are active ingredients registered for the control of this disease, there is little study about products with alternatives for the control of the fungus *S. lycopersici*. Thus, the objective of this work was to study the effect of alternative products in controlling the septoriose. The rationale for the study was to find efficient products that are less harmful to the environment. The study was conducted at the experimental station of EPAGRI in the state of Santa Catarina, Brazil. Twelve products were tested to control *Septoria*: *Bacillus subtilis* QST 713 (274 mg/L a.i.), *Bacillus subtilis* QST 713 autoclaved (274 mg/L a.i.), lime sulfur (10,000 mg/L c.p.), benzalkonium chloride (250 mg/L a.i.), mixed mineral fertilizer (2,000 mg/L c.p.), sodium hypochlorite (320 mg/L a.i.), peracetic acid (5,440 mg/L a.i.), Bordeaux mixture (3,000 mg/L c.p.), Viçosa mixture (3,000 mg/L c.p.), *Trichoderma harzianum* Rifai ESALQ-1306 (600 mg/L a.i.), acibenzolar-S-methyl (25 mg/L a.i.), potassium phosphite (2,000 mg/L a.i. with 1,340 mg/L phosphorous acid) and biostimulant (200 mg/L c.p.). The doses used were based on label, previous tests *in vitro* and in

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phytotoxicity events in tomato plants. In the *in vitro* experiments, the products that were not able to promote the formation of an inhibition halo were: biostimulant, potassium phosphite, acibenzolar-S-methyl, *Trichoderma harzianum*, Viçosa mixture and Bordeaux mixture. The products *B. subtilis*, lime sulfur, benzalkonium chloride, mixed mineral fertilizer, peracetic acid, sodium hypochlorite and autoclaved *B. subtilis* were able to inhibit fungal growth *in vitro*, forming a halo of inhibition. The chemical fungicides mancozeb + pyraclostrobin + fluxapyroxad were used as a positive control. *In vivo*, the positive control was able to control 100% of the incidence and severity of *Septoria* and no symptoms were observed in the plants. For incidence, the products that controlled at least 80% of the disease were lime sulfur and mixed mineral fertilizer. When considering the disease severity, the products that controlled at least 80% of the disease were: lime sulfur, mixed mineral fertilizer, *Bacillus subtilis* QST713 and benzalkonium chloride. The products Bordeaux mixture, Viçosa mixture, sodium hypochlorite and peracetic acid caused phytotoxicity when applied to tomato plants. Although lime sulfur has shown promise, its successive application can lead to a decrease in the photosynthetic rate.

Keywords: Chemical control; Solanum lycopersicum; tomato disease; Septoria lycopersici; septoria leaf spot.

1. INTRODUCTION

The septoriose was reported for the first time in Argentina in 1882 [1] and nowadays occur anywhere in tomato crops [2]. *S. lycopersici* infect tomato leaves by both stomata and direct penetration [3]. Its importance depends on the favourable weather conditions, which occur when the relative humidity is above 85%, temperature is between 20 to 25ºC [4] and foliar wetting periods greater than 20h [5]. Symptoms appear one week after inoculation, and after six weeks, defoliation is close to 100%, and losses are significant due to the sunscald on tomato fruits [6], when in humid conditions and no control measures are used [7]. In each cultivation cycle, the disease starts in the leaves of the shallows due to the raindrops that fall on fragments of plants with *Septoria* spores and cause splashes spreading the spores to the surrounding tomato leaves [8].

Extensive prior research has focused on the chemical control of Septoria, revealing the potential efficacy of various active ingredients and diverse approaches for managing tomato *Septoria.* [9]. To explore alternative products, additional testing is required for the control of septoriose.

One of the alternatives for the control of *Septoria lycopersici* is the use of biological agents, such as beneficial bacteria and fungi. Studies have shown that the application of *Bacillus subtilis*, for example, can significantly reduce disease severity in tomato plants [10,11,12]. Likewise, the pulverization of *Trichoderma* spp. has also shown promising results in disease control [10,13,14]. Another alternative is the use of potassium phosphite. Studies have shown that the application of potassium phosphite can significantly reduce disease severity in tomato plants [15,16,17]. In addition, some chemical compounds have been shown to be effective in controlling tomato plant diseases, such as sodium hypochlorite [18,19] and mixture formulations, such as Bordeaux mixture, lime sulfur and Viçosa mixture [20,21].

Thus, the use of biological agents and organic and sanitizing compounds may be an efficient alternative for controlling *Septoria lycopersici* in tomato plants, provide a more sustainable option. The objective of this work was to study the effect of alternative products in controlling the septoriose in tomato plants compared to a combination of three good fungicides to control septoriose (pyraclostrobin + fluxapyroxade + mancozeb) as a positive check.

2. MATERIAL AND METHODS

2.1 Molecular Identification

The isolate of *S. lycopersici* were identified by sequencing the Internal Transcribed Spacer (ITS gene), and the sequence were deposited at the GenBank. The DNA was extracted via PureLink Genomic DNA Mini Kit and PCR was performed in thermocycler TC-9639 (Loccus) with the following program: 94ºC for 4 min followed by 30 cycles of 94ºC for 30 sec, 55ºC for 1 min, 72ºC for 1:30 min, and a final extension at 72ºC for 10 min. PCR product were analyzed by agarose gel (0.7%) electrophoresis in 0.5x TBE buffer conducted at 90V for 1h and scanned using the

imaging system L-PIX EX (Loccus) and sent to sequencing.

2.2 Assessing Safe Doses of Viçosa Mixture, Bordeaux Mixture, and Lime Sulfur

The Viçosa syrop, the Bordeaux mixture and the lime sulfur were applied at doses of 1,000, 2,500, 5,000, 10,000, 20,000, 30,000, 40,000 and 50,000 mg/L in tomato plants. After 72 h, the plants were evaluated for phytotoxicity symptoms to determine safe doses for application on tomato plants in our conditions. In the case of lime sulfur, IRGA (Infra-Red Gas Analyser) (Li-Cor) was used to measure whether the photosynthetic rate decreased as a result of three successive applications without interference from rainfall. The interval of one week elapsed between each pulverization, and after this period, the photosynthetic rate was measured using an IRGA. The results were submitted to analysis of variance, when significant by the F test, the means were compared by the Scott-Knott statistical test at 5% of probability ($P \leq 0.05$) using Sisvar software.

2.3 Inhibition of *Septoria lycopersici in vitro*

The experiment was conducted in Petri dishes of 9 cm and three replicates per treatment. After pouring the malt extract culture media (malt extract 20 g/L and agar 20 g/L), 100 µL of a spore suspension at 10^5 spores per ml was spread over the culture media surface using a Drigalski handle. In the center of the Petri dish was placed 10 µL of the fungicides solution at the recommended dose. Fungicides doses were employed as describe in their labels (Table 1) or according to preliminary efficiency tests. The inhibition halo was measured after 14 days of incubation at 25ºC and a photoperiod of 12h. Mixed mineral fertilizer is composed by acetic acid, sodium molybdate and nickel sulfate. Bioestimulant is composed by 25% L-glutamic acid and 4% of soluble nitrogen. Phosphite is composed phosphorous acid 67% and K_2O 20%.

2.4 Inhibition of *Septoria lycopersici in vivo*

Tomato plants were cultivated in vessels with five-litre of capacity. When plants reach four leaflets completely developed, fungicides at the label doses (Table 1) were sprayed until the rain off point (1,000L/ha). The water used to prepare the fungicides solutions had pH 7. After one hour from the fungicides spraying, a spore suspension at 10^5 spores/mL was sprayed over plants. Plants were incubated in a greenhouse for 14 days. After this period, the incidence and severity of the disease were evaluated using a diagrammatic scale for *S. lycopersici* of the tomato [22]. The experiment was replicated two times. The data were transformed to the percentage of the control. A product was considered efficient when it was able to control above 80% of disease incidence and severity compared to the check (without fungicide

Table 1. Fungicides doses used in this study

Fungicides	Doses - ppm or $(\mathbf{mg/L})$
Chemical fungicide (positive control) - Pyraclostrobin + Fluxapyroxade + Mancozeb	116.55 + 58.45 + 4,000 a.i.[2]
Bacillus subtilis (13.68 g/L)	274 a.i.
Lime sulfur $(50\%\ S; 5\%Ca)$	10,000 c.p. [3]
Benzalkonium chloride $(100\ g/L)$	250 a.i.
Mixed mineral fertilizer (acetic acid, sodium molybdate, nickel nitrate)	2,000 c.p.
Peracetic acid $(17 \pm 0.98\%)$	5,440 a.i.
Sodium hypochlorite ($2 - 2.5\%$ active chlorine)	320 a.i.
Bacillus subtilis (13.68 g/L) autoclaved	274 a.i.
Bordeaux mixture $(20\%Cu, 10\%\ S, 3\%Ca)$	3,000 c.p.
Viçosa mixture $(8\%\ K_2O, 0.8\%Mg, 8\%\ S, 3.5\%\ B, 9\%Cu, 3\%Zn)$	3,000 c.p.
Trichoderma harzianum $(48\ g/L)$	600 a.i.
Acibenzolar-S-metil $(500\ g/kg)$	25 a.i.
Potassium phosphite $(67\%$ phosphorous acid, $20\%\ K_2O)$	2,000 c.p. $(1{,}340$ a.i. [1]$)$
Biostimulant (25% L-glutamic acid, 4% of soluble nitrogen)	200 c.p.

[1] Refers to the quantity of phosphorous acid. [2] a.i. means active ingredient. [3] c.p. means commercial product

pulverization). A positive control (Table 1) was used as one of the best treatment to tomato septoriose [9]. The results were submitted to analysis of variance, when significant by the F test, the means were compared by the Scott-Knott statistical test at 5% of probability (P≤0.05) using Sisvar software.

3. RESULTS AND DISCUSSION

3.1 Molecular Identification

The isolate used herein identified as *S. lycopersici* was deposited in the Genbank with the code ON890816.

3.2 Phytotoxicity Assessment using Viçosa mixture, Bordeaux mixture and lime sulfur

The lime sulfur did not cause visual phytotoxicity at a dose up to 30,000 mg/L. Viçosa mixture and Bordeaux mixture did not cause visual phytotoxicity up to doses of 2,500 mg/L and 1,000 mg/L, respectively (Fig. 1).

The successive application of lime sulfur at a dose of 30,000 mg/L decreases photosynthesis from the second application onwards in an environment without rain (Table 2). Lime sulphur, again, did not cause visual phytotoxicity to tomato plants at the tested dose.

3.3 Inhibition Halo Due to Product Application *In Vitro*

Among the 12 product tested, six commercial products caused inhibition halo showing the dose used was able to control the spore germination which indicates efficacy of the alternative product and also an adequate dose to control the pathogen *in vitro* (Fig. 2 and 3). Only acibenzolar-S-metil, bioestimulant, Bordeaux mixture, potassium phosphite, *Trichoderma harzianum* and Viçosa mixture were unable to prevent spore germination and form an inhibition halo (Fig. 2 and 3).

3.4 Inhibition of S. lycopersici *In Vivo*

Considering the percentage of incidence control, only lime sulfur and mixed mineral fertilizer were able to control more than 80% of the disease incidence. As for the severity control, only lime sulfur, mixed mineral fertilizer and benzalkonium chloride were able to control more than 80% of the disease severity, obtaining results equal to the positive control (mancozeb + fluxapyroxad + pyraclostrobin) (Fig. 4).

The products peracetic acid (5,440 mg/L), bordeaux mixture (3,000 mg/L), Viçosa mixture (3,000 mg/L), and sodium hypochlorite (320 mg/L a.i.) caused phytotoxicity after 72 hours of application in tomato plants (Fig. 5). Hence, we do not endorse its utilization.

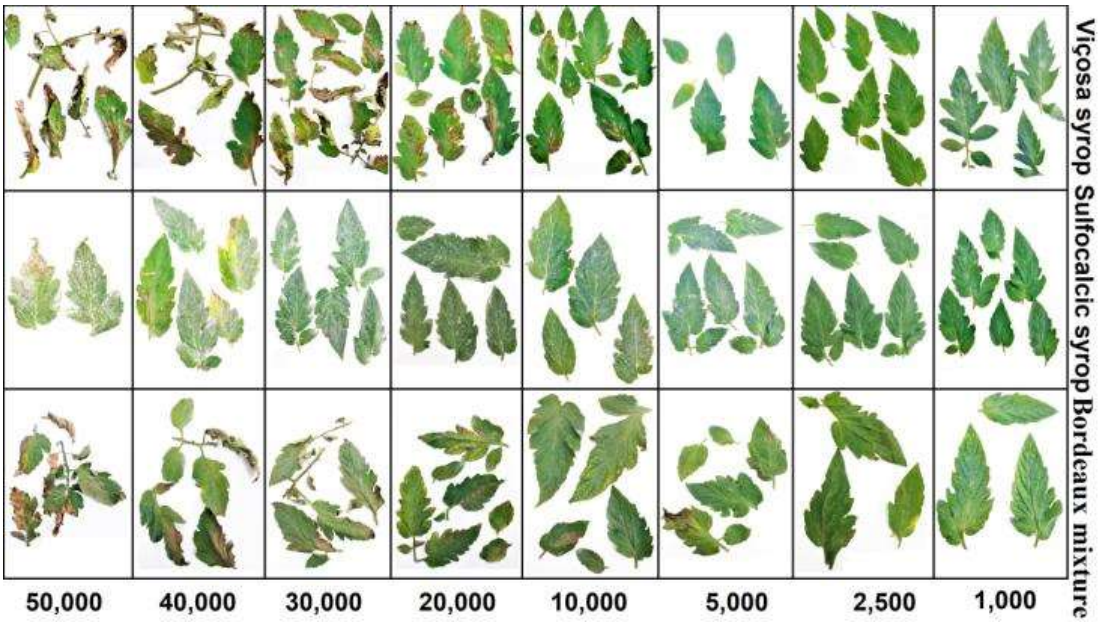

Fig. 1. Visual Phytotoxicity of the Bordeaux mixture, Viçosa and Sulfocalcic syrops (lime sulfur) in range of concentration (mg/L)

Table 2. Effect of successive applications of lime sulfur on the photosynthetic rate of tomato leaves at the dose of 30,000 mg/L

	Lime sulfur	µmol $CO_2/m^2/s$*	CV (%)
1º Spraying	Without fungicides	21.72±2.43 a	4.10
	With fungicides	21.04±0.90 a	
2º Spraying	Without fungicides	21.62±2.22 a	4.81
	With fungicides	18.76±1.75 b	
3º Spraying	Without fungicides	20.23±1.65 a	6.10
	With fungicides	16.29±2.71 b	

** Unit used to indicate photosynthesis*

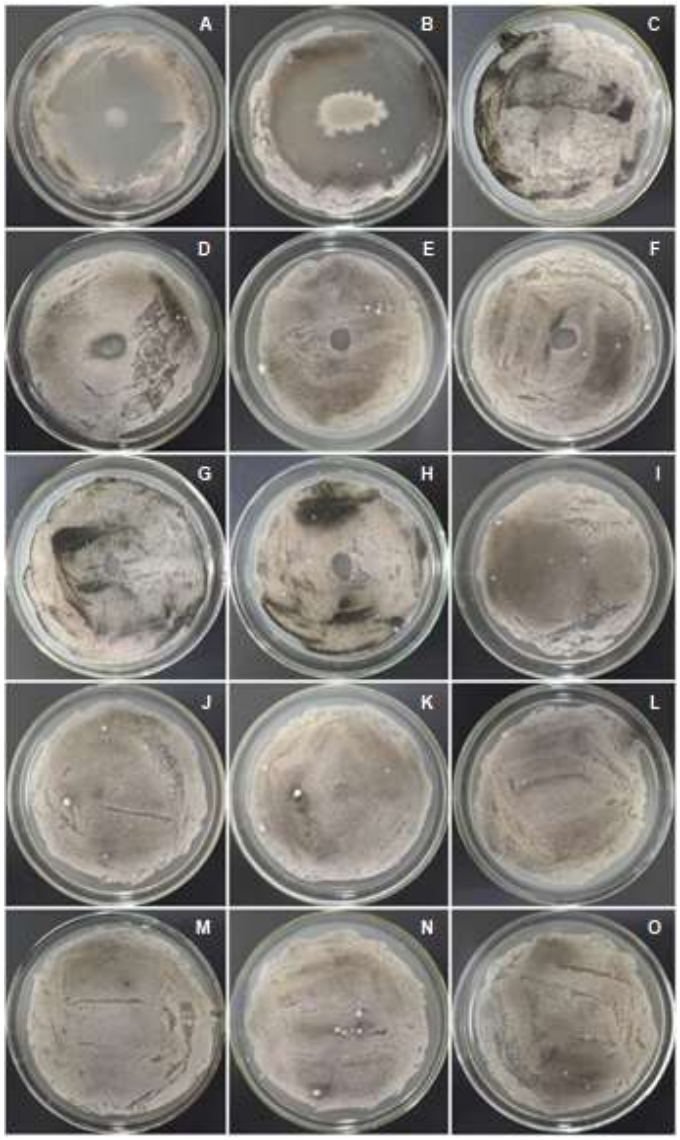

Fig. 2. Effect of the fungicides on the inhibition halo formation against *Septoria lycopersici*

A – Pyraclostrobin + Fluxapyroxade + Mancozeb (116.55 + 58.45 + 4,000 mg/L a.i.). B – Bacillus subtilis QST 713 (274 mg/L a.i.). C – Bacillus subtilis QST 713 autoclaved (274 mg/L a.i.) D – Lime sulfur (10,000 mg/L c.p.). E – Benzalkonium chloride (250 mg/L a.i.). F – Mixed mineral fertilizer (2,000 mg/L c.p.). G – Sodium hypochlorite (320 mg/L a.i.). H – Peracetic acid (5,440 mg/L a.i.). I – Bordeaux mixture (3,000 mg/L c.p.). J – Viçosa mixture (3,000 mg/L c.p.). K – Trichoderma harzianum Rifai ESALQ-1306 (600 mg/L a.i.). L – Acibenzolar-S-metil (25 mg/L a.i.). M – Potassium phosphite (2,000 mg/L c.p. with 1340 mg/L phosphorous acid a.i.). N – Bioestimulant (200 mg/L c.p.). O - Check

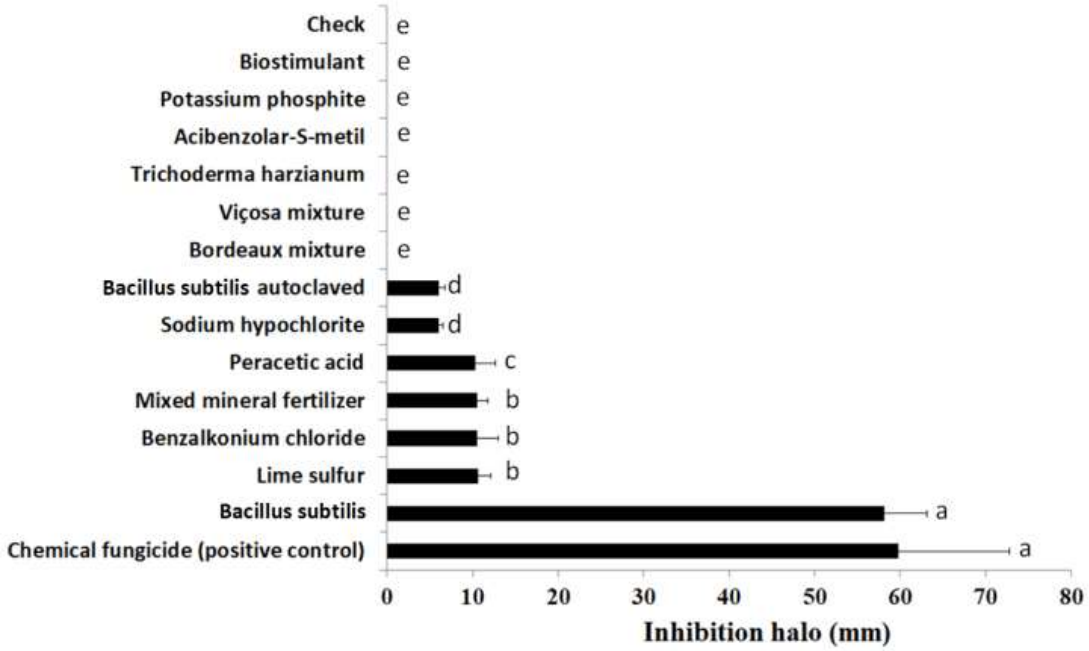

Fig. 3. Inibition halo promoted according to the fungicides at their label doses against *S. lycopersici* growing in malt-extract *in vitro*

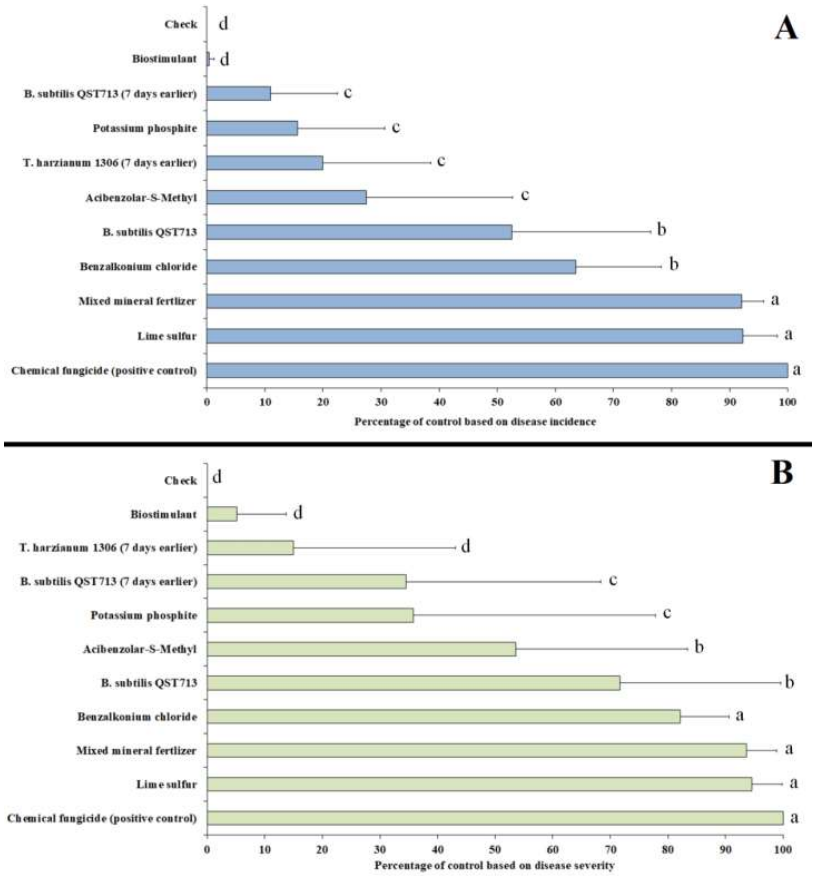

Fig. 4. Effect of alternative products on the incidence (A) and severity of tomato septorioses (B) considering two experiments

Fig. 5. Visual phytotoxicity of some products after 72 h of spraying on tomato plants
A - Peracetic acid (5,440 mg/L a.i.). B - Bordeaux mixture (3,000 mg/L c.p.). C – Viçosa mixture (3,000 mg/L c.p.). D - Sodium hypochlorite (320 mg/L a.i.). The black arrows in C and D indicate the locations where visible symptoms of phytotoxicity were observed

3.5 Discussion

Comparing the *in vitro* results of the application of *B*. *subtilis* and autoclaved *B*. *subtilis*, it is noted that as the bacteria was alive, in the first case, it used the culture medium to produce fungicidal compounds that formed a considerable halo of inhibition. In the second case, in which the bacteria present in the product died due to the autoclaving process, it is noted that there was no bacterial growth in the culture medium, but even so, an inhibition halo was formed, indicating the existence of thermostable molecules in the formulated product. In the *in vivo* experiment, the application of *B*. *subtilis* one hour before the application of the *S*. *lycopersici* spore suspension decreased the incidence and severity of septorioses by 50% and 70%, respectively. For the bacteria applied seven days before the application of *S*. *lycopersici* spores, the result was more modest and with greater variation, supporting the idea that the metabolites produced by the bacteria during the fermentation process for making the product play an important role in the control of the disease. In this application of *B*. *subtilis* seven days before, the colonization of the leaf by the bacteria and the use of the nutritional resources of the leaf for survival and production of metabolites *in loco* do not seem to happen, and it is better to apply the biological product on the days most prone to penetration by the fungus.

Efficient control is closely related to the presence of fungicidal molecules produced during the product's fermentation process, among other factors. It was demonstrated that the application of *B*. *subtilis* significantly reduced the severity of the disease in tomato plants when applied at intervals of 15 days [23]. Although the use of bacteria such as *B*. *subtilis* is promising for controlling foliar diseases in tomato, in practice its use is greatly affected by the large number of applications of copper-based products that also kill the bacteria considered agents of biological control [24]. Therefore, the use of *B*. *subtilis* to control septoriosis and other diseases only makes sense if it is adopted 100% in a greenhouse or thermoregulated environment that does not involve the application of any chemical products harmful to those bacteria including mancozeb or any multisite fungicide and copper-based produts, and also in organic systems that do not use copper-based products.

Regarding *T*. *harzianum*, an efficient control of *Septoria* was not observed both *in vitro* and *in vivo* results. *In vitro*, we tested the following doses 12,500, 25,000, 50,000, 100,000, 200,000 and 400,000 mg/L but none of them formed an inhibition halo (data not shown). In the *in vivo* experiments, it was observed that the application of the product led to a slight deformation in the tomato leaf blade, which may be due to the formulation of the product used. However, there

are several reports of success in the use of *Trichoderma* to control *Septoria* [23] and other diseases in tomato crops [25,26, 27].

In this work, potassium phosphite (2,000 mg/L a.i. with 1,340 mg/L phosphorous acid) was not considered efficient. Looking for some direct effect on *S*. *lycopersici* we tested *in vitro* potassium phosphite doses 2,000, 4,000, 8,000, 16,000, 32,000 and 64,000 mg/L, but none of them formed an inhibition halo (data not shown). However, in some specific situations, studies have shown positive effects from the use of phosphite in controlling other pathogens in tomato crops [28]. Foliar pulverization of phosphite on tomato plants can be used to activate plant-defense responses, but they would not contribute to improve growth and nutritional status of tomato plants, indicating that phosphite is not a relevant source of nutrients [29].

Acibenzolar-S-methyl, a resistance inducer, was also not considered efficient in controlling tomato septorioses. The inefficiency of acibenzolar-S-methyl was also reported against *Xanthomonas hortorum* pv. *gardneri* and *Xanthomonas euvesicatoria* pv. *perforans* [28]. Another author reported significant control of bacterial leaf spot, however, reported that acibenzolar-S-methyl (35 mg a.i./liter) was phytotoxic, causing stunting, chlorosis, epinasty, narrowing of leaf blades, and defoliation [30].

Sodium hypochlorite caused phytotoxicity in tomato plants at the dose that controlled *Septoria* germination and mycelial growth *in vitro*, and therefore, its foliar application is not recommended. However, sodium hypochlorite may have some effect to control soil pathogens [31].

The Bordeaux mixture and Viçosa mixtures at the tested doses did not promote the inhibition halo, however, even at a low dose (3,000 mg/L) it caused phytotoxicity in tomato plants. For this reason, the incidence/severity of the disease was not accounted for, and its use in the treatment of this disease is not indicated. Furthermore, the use of a lower dosage to avoid phytotoxicity would not control the pathogen, and would not guarantee the health of the plants. The physiological effect due to the application of Bordeaux mixture and Viçosa mixture in smaller doses has not been studied, but cannot be discarded. However, there are reports of successful use in tomato crops without mentioning the occurrence of phytotoxicity [32,21,20]. For Viçosa mixture there are also reports of success in the tomato crop using the dosage of 4,000 mg/L to control powdery mildew without reports of negative effects on the crop [33,21]. There seems to be considerable variation in how Viçosa and Bordeaux mixtures are prepared, or even between the preparation of those mixtures and the use of ready-to-use mixtures, since in some cases there is phytotoxicity and in others, even using higher doses, there are no reports. The use of Viçosa and Bordeaux mixtures at 1% (10,000 mg/L) in the tomato crop without events of phytotoxicity was reported [34], and in our conditions phytotoxicity effects were observed at doses of 0.3–0.5% and 0.25%, respectively. Despite the excellent performance of lime sulfur at 10,000 mg/L in the control of septoriosis demonstrated herein, we do not know if the interference in photosynthesis affects production or if there is some plant compensatory mechanism.

In the doses studied herein, the benzalconium chloride and mixed mineral fertilizer based on acetic acid did not shown any deleterious effect on tomato plants and could be used as fungicide of low enviromental impact. However, its usage must be approached cautiously, as it exhibits reactivity and has the potential to inflict harm upon the hands. Previous studies have shown that the application of sanitizers as benzalconium chloride can reduce the incidence of *S*. *lycopersici* in tomato plants [9]. The acetic acid are known as inhibitor of food-borne pathogens [35], and its use have been reported in unusual way to control plant pathogen and pests [36,37]. On the other hand, despite of potential of peracetic acid as disinfectant of low envinromental impact it can have phytotoxic effects [38]. Another researcher's report highlights that peracetic acid proved more efficient than sodium hypochlorite [39]. In our research, both peracetic acid and sodium hypochlorite causes phytotoxic effects on tomato plants at the dose at which the inhibition halos were formed *in vitro* against *S. lycopersici*. For sodium hypochlorite and peracetic acid, doses less than 160 mg/L a.i. and 2,720 mg/L a.i, respectively, did not form a halo of inhibition against *S*. *lycopersici* (data not shown).

4. CONCLUSION

Peracetic acid, Bordeaux mixture (0.3%), Viçosa syrup (0.3%), and sodium hypochlorite led to phytotoxicity. Promising outcomes were observed with products containing *B*. *subtilis*

QST713, benzalkonium chloride, mixed mineral fertilizer, and lime sulfur. The studied *B*. *subtilis* QST713-based product exhibited a direct effect against *Septoria lycopersici*.

ACKNOWLEDGEMENTS

The authors are thankful to the agricultural research and rural extension enterprise of santa catarina (epagri) and the foundation for research and innovation support of the state of santa catarina (fapesc) for financing and encouraging the execution of the project that gave rise to this publication. In addition, we would like to thanks the research assistant daniela alves cardoso for the help during the execution of the experiments.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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