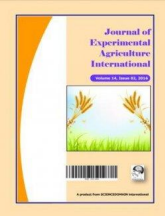

Journal of Experimental Agriculture International

33(4): 1-9, 2019; Article no.JEAI.48027
ISSN: 2457-0591
(Past name: American Journal of Experimental Agriculture, Past ISSN: 2231-0606)

Phosphite-based Products in the *In vitro* *Colletotrichum musae* Control

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Authors' contributions

This work was carried out in collaboration among all authors. The author MLMR contributed to the idea of the work, wrote the manuscript and collaborated in the execution of the work and evaluations. Author EHM contrived with the laboratory and reagents for analysis. The authors PJLP and PVLD contributed in the execution of the experiment. The author RCFR contributed with statistical analysis. The author JMDSP contributed with the statistical analyzes and correction of the manuscript. The author GPM contributed in providing raw material for the execution of the work. The authors MBF and LSS contributed in the execution and bibliographical research. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JEAI/2019/v33i430148
Editor(s):
(1) Dr. Slawomir Borek, Professor, Department of Plant Physiology, Adam Mickiewicz University, Poland.
Reviewers:
(1) Martín María Silva Rossi, Estudio Agronómico, Santa Fé Argentina, Argentina.
(2) Ana-Maria Andrei, Research Development Institute for Plant Protection, Romania.
(3) Ferenc Bagi, University of Novi Sad, Serbia.
(4) Mónica Guadalupe Lozano Contreras, National Institute of Forest Research Agricultural and Livestock (INIFAP), Mexico.
Complete Peer review History: http://www.sdiarticle3.com/review-history/48027

Original Research Article

Received 08 January 2019
Accepted 23 March 2019
Published 01 April 2019

ABSTRACT

Aims: The aim of this study was to evaluate the *in vitro* effect of different phosphite formulations and concentrations on the development of *Colletotrichum musae*. Sample: to evaluate the inhibition of germination, mycelial growth and sporulation of *Colletotrichum musae*.
Study Design: Treatments were conducted in a completely randomized design, with 4 replicates, each replicate consisting of 1 Petri dish.

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Place and Duration of Study: Laboratory of Post-Harvest Pathology, State University of Montes Claros, between March and October 2017.
Methodology: Three different phosphite formulations were used: FCu1 (4% Cu + 20% P_2O_5), FCu2 (4% Cu + 22% P_2O_5) at concentrations of 0.5;1.0; 1.5 and 2.0 mL L^{-1} and FK (42% P_2O_5 + 27.7% K_2O) at concentrations of 0.5; 1.0; 1.5 and 2.0 mg.L^{-1}. Products were incorporated into the respective culture media. Culture medium alone and culture medium + imazalil were used as controls. Petri dishes were housed in BOD chamber at 25°C under a 12 hours photoperiod.
Results: Results were submitted to analysis of variance and regression, and means were compared by the Tukey test (P <0.05). Control was compared to the other treatments by the Dunnet's test (P <0.05). Among the tested phosphite formulations, copper and potassium phosphites were found to reduce the mycelial growth of *Colletotrichum musae*. FCu2 presents a fungicide-like effect from the concentration of 0.5 m.L^{-1} in the control of conidia production. As for the FCu1, a fungicide-like effect was observed in the control of germination from the concentration of 1.5 mL.L^{-1}.
Conclusion: A significant fungistatic effect was observed between the concentrations of the products in the mycelial growth, sporulation and germination obtaining control of up to 100% of the development of *C. musae*. Copper phosphites were as effective as fungicide in inhibiting fungal development.

Keywords: Disease; anthracnose; alternative treatment.

1. INTRODUCTION

Anthracnose caused by *Colletotrichum musae* (Berk & Curt.) von Arx. (Teleomorph: *Glomerella musarum* Petch) stands out as the most important post-harvest disease in banana crops. Fruits infected by the fungus have accelerated maturation, an undesirable aspect for consumers, which makes them unviable for export. Losses due to anthracnose can reach up to 80%, causing various damages to fruits [1].

Among the methods for controlling the disease, the most important are cultural and chemical control with the use of Tiabendazol and Imazalil fungicides [2,3]. The latter method presents as negative effects damage to the environment and the emergence of fungal isolates resistant to fungicidal molecules. In addition, they may affect human health since they can leave residues in fruit pulp [4,5,6].

The excessive use of agrochemicals in fruit trees is a growing worldwide concern with real possibilities of environmental contamination. Thus, the demands of the consumer market for high-quality fruits produced with the substitution of pollutant and non-renewable inputs are increasing [7]. There are several alternative strategies for controlling diseases such as modified atmosphere [8], use of essential oils and extracts [9,10,11] to control diseases caused by phytopathogenic fungi.

Phosphites are substances originating from the neutralization reaction of phosphorous acid by a base [12]. These products have been used in agriculture to stimulate plant growth for presenting direct action on pathogens or for inducing defense mechanisms, being presented as an alternative to the application of fungicides [13,14,15].

In vitro results confirm the effect of potassium phosphite on the reduction of the mycelial growth of *C. musae* by 84% when comparing with control [10]. The application of phosphites in other species of the genus *Colletotrichum* as well as other genus of fungi has been investigated by several authors. Lopes et al. [16] observed that magnesium and potassium phosphites inhibited 50% of the mycelial growth of *Colletotrichum gloeosporioides*. Alexandre et al. [17] found that potassium phosphite showed fungitoxic activity in the development of *Colletotrichum tamarilloi*. In *Fusarium solani*, potassium phosphite inhibited growth and mycelial density with the application of 50 ppm [18].

The effect of phosphite application on the management of plant diseases may vary according to the type of phosphite, dose applied and target pathogen. There are reports of partial and / or total inhibition of mycelial growth, conidial production / germination, and appressorium formation of different fungi [19,11, 20,17,10,21]. In this sense, the aim of this study was to evaluate the *in vitro* effect of different

phosphite formulations and concentrations on *C. musae* development.

2. MATERIALS AND METHODS

Experiments were carried out at the Laboratory of Post-Harvest Pathology, State University of Montes Claros, Minas Gerais, MG. *C. musae* isolate was obtained from bananas purchased in a commercial growing area, which were selected for showing dark spots and mass of orange conidia, typical anthracnose symptoms.

For the *in vitro* sensitivity assessment copper phosphites FCu1 (4% Cu + 20% P_2O_5), copper FCu2 (4% Cu + 22% P_2O_5) at concentrations of 0.5; 1.0, 1.5 and 2.0 $mL.L^{-1}$ and potassium phosphite FK (42% P_2O_5 + 27.7% K_2O) at concentrations of 0.5, 1.0, 1.5 and 2.0 $mg.L^{-1}$ were incorporated into melting BDA medium and poured on to Petri dishes of 9 cm in diameter. After solidification of the culture medium, a 5 mm diameter mycelium disc with 7 days of culture was transferred to the center of dishes containing treatments. BDA culture medium alone and BDA + Imazalil medium (0.5 $mL.L^{-1}$) were used as controls. The sides of dishes were sealed with clear plastic film to avoid possible evaporation of compounds and drying of the culture medium. Petri dishes were housed in BOD chamber at 25ºC under a 12 hours photoperiod. Measurements were carried out by means of daily measurements of the diameter of colonies (average of the two diametrically opposed measurements), 24 hours after the beginning of the experiment, always at the same time and ending when the mycelial growth of control reached the edge of the dish. Data were used to calculate MGRI (Mycelial Growth Rate Index) in mm / day, using the following formula [22]: Σ MGRI = (D - Da) / N, where: D: Current mean diameter; Da: Previous mean diameter; N: number of days after pricking.

When the mycelial growth of control (absence of phosphite) reached the entire dish, conidia production was evaluated. For this, 50 mL of distilled sterile water were added to each Petri dish using the Drigalski loop, colonies were scraped for the release of conidia. The spore suspension was filtered through a double layer of sterile gauze. Then, 500 µL of each suspension was removed and placed in Newbauer chamber, where conidia were counted using an optical microscope and spore counter.

The effect of phosphites on conidia germination at the same concentrations previously used was also verified. Treatments were added to the agar-melting water medium, and then the medium was poured onto 9 cm diameter Petri dishes. After solidification, 1 mL of the conidia suspension at concentration of 2.5 x 10^5 *C. musae* spores / mL was placed on the agar-water medium and spread with the aid of the Drigalsk loop. Dishes were taken to BOD chamber at 25°C under a 12 hours photoperiod for 15 hours. Then, they were taken to the refrigerator to stop germination. The germination rate was determined by counting germinated spores of the fungus. Conidia presenting the length of the germinative tube greater or equal to the conidia diameter were considered germinated.

The design was completely randomized in a 3x4 + 2 factorial scheme, with three phosphate formulations (FCu1, FCu2 and FK), four concentrations (0.5; 1.0; 1.5 and 2.0 $mL.L^{-1}$) and controls (absence of treatment and Imazalil fungicide). Four replicates were used per treatment, each replicate consisted of a Petri dish. Data were submitted to analysis of variance and the means compared by the Tukey test at 5% probability. Controls were compared to the other treatments by the Dunnet's test at 5% probability. Analyses were performed using the R software [23].

3. RESULTS AND DISCUSSION

Significant interaction (P <0.05) was observed among sources of phosphite at different concentrations used for all characteristics evaluated. However, as there was no adjustment of regression models, and the mean values of MGRI, sporulation and germination of *C. musae* conidia were compared by the Tukey test (P<0.05) and controls were compared to the other treatments by the Dunnet's test (P <0.05).

Table 1 shows that at concentration of 0.5 $mL.L^{-1}$, the lowest MGRI was promoted by FCu1 and FK. At concentrations of 1.0, 1.5 and 2.0 $mL.L^{-1}$, the sources that promoted the greatest reduction in MGRI were FCu1 and FCu2.

These results demonstrate the efficiency of phosphites in controlling the development of *C. musae*. In this way, different sources of copper and potassium phosphite are able to control different phytopathogens (*Pythium, C. gloeosporioides, Monilinia frutícola* e *Colletotrichum tamarilloi*) [24,25,26,18,17].

It was verified that in treatments using FCu1 and FCu2 from concentration of 1.0 mL.L^{-1} were as efficient as Imazalil fungicide, showing 100% inhibition of fungal mycelial growth (Table 1).

In treatment using FK, only the lowest concentration showed fungistatic effect on *C. musae*. However, Nojosa et al. [27] observed the opposite effect, reporting in their work that potassium phosphite inhibits 62,26% the mycelial growth of *Phoma costarricensis* Echandi in coffee tree at the highest applied concentration (10.00 mL. L^{-1}).

Inhibition of pathogen development by phosphite-based products may occur due to the direct action of the product on the fungus, acting in the process of oxidative phosphorylation as observed in Oomycetes [28]. The direct action of phosphites on pathogens under *in vitro* conditions can be verified even at doses lower than those recommended by the manufacturer [10,29].

In the comparison of treatments to controls by means of the Dunnet's test at 5% probability, all sources associated to all concentrations reduced MGRI (Table 2).

In comparison with the fungicide, it was verified that FCu1 and FCu2 at concentrations of 1.0 mL.L^{-1},1.5 mL.L^{-1} and 2.0 mL.L^{-1} did not differ significantly, which shows that such sources associated with these concentrations were as efficient as Imazalil.

As the phosphite concentration increases, better results are obtained, as can be verified in other patossystems [16,22,30,31].

Copper phosphites present different results according to the fungal species. Dantas et al. [21] obtained control of the mycelial growth of *Lasiodiplodia theobromae* when applying copper phosphite at concentration of 2.0 mL.L^{-1}, but the same control was not obtained when applying in *Alternaria* sp, *Colletotrichum gloeosporioides*, *Fusarium* sp., *Rhizopus* sp.

Borin et al. [32] found that copper phosphite showed positive effect on the reduction of 52% to 76% and 82% to 96% of the radial growth of *Fusarium verticilliodes* and *F. graminearum* isolates, respectively, using copper phosphite when comparing with control.

The success of the application of phosphites in the control of fungal development occurs due to the reduction of aerobic respiration, affecting ATP production, which is the source of energy responsible for the development of *C. musae* [24].

FK showed no significant difference among concentrations tested in both controls. Oliveira et al. [10] disagree with this result, since the authors reported that potassium phosphite at concentration of 3µL.mL^{-1} inhibited the mycelial growth of *C. musae* by 91.8% compared to the absence of treatment.

The results obtained by Spolti et al. [33] disagree with those obtained in this experiment; the authors verified that the mycelial growth rate index (MGRI) presented inverse quadratic order relationship to potassium phosphite in the culture medium.

The antisporulating effect of phosphites on *C. musae* is shown in Table 3. At concentration of 0.5 mL.L^{-1}, the lowest sporulations were observed when FCu2 and FK were used. At concentration of 1.0 mL.L^{-1}, the three sources of phosphite promoted the same effect.At concentrations of 1.5 and 2.0 mL.L^{-1}, FCu1 and

Table 1. Mycelial growth rate index (MGRI) of *Colletotrichum musae* submitted to different phosphite concentrations

Sources of Phosphite	**Concentrations**			
	0.5 mL.L^{-1}	**1.0 mL.L^{-1}**	**1.5 mL.L^{-1}**	**2.0 mL.L^{-1}**
F Cu 1	0.75 aB	0.0 aA	0.0 aA	0.0 aA
F Cu 2	1.5 bB	0.0 aA	0.0 aA	0.0 aA
	Concentrations			
	0.5 mg.L^{-1}	**1.0 mg.L^{-1}**	**1.5 mg.L^{-1}**	**2.0 mg.L^{-1}**
F K	0.25 aA	0.75 bAB	1.0 bB	1.25bB
Absence of phosphite	3.23			
Imazalil	0.0			
CV (%)	72.73			

Means followed by the same lowercase letter in the column and uppercase in the row do not differ by the Tukey's test (P <0.05)

Table 2. Means of treatments compared by the Dunnet's test for the following variables: mycelial growth rate index (MGRI), sporulation and germination of *Colletotrichum musae* conidia submitted to different phosphite concentrations

Sources of Phosphite		MGRI	Sporulation	Germination (%)
FCu1	**0.5**	**0.75 x y**	**41.50 x y**	**89.25 y**
	1.0	0.0 x	30.25 x y	39.50 x y
	1.5	0.0 x	0.00 x	3.25 x
	2.0	0.0 x	0.00 x	0.25 x
FCu2	**0.5**	**1,5 x y**	**6.00 x**	**48.25 x y**
	1.0	0,0 x	12.00 x	22.00 x y
	1.5	0.0 x	8.25 x	48.25 x y
	2.0	0.0 x	4.50 x	37.00 x y
FK	**0.5**	**0.25 x y**	**10.00 x**	**96.50 y**
	1.0	0.75 x y	34.50 x	90.50 y
	1.5	1.0 x y	46.25 x y	88.25 y
	2.0	1.25 x y	92.25 y	91.00 y
Absence of phosphite		3.23	102.00	100.00
Imazalil		0.00	0.00	0.00

Averages of treatments followed by the letter X and means of treatments followed by the letter Y differ statistically from control, absence of phosphite and Imazalil by the Dunnet's test (P <0.05), respectively

Table 3. Production of *Colletotrichum musae* conidia submitted to different sources of phosphite at different concentrations

Sources of phosphite	Concentrations			
	0.5 mL.L^{-1}	**1.0 mL.L^{-1}**	**1.5 mL.L^{-1}**	**2.0 mL.L^{-1}**
F Cu 1	41.5bB	30.25 a AB	0.0 aA	0.0 aA
F Cu 2	6.0 aA	12.0 aA	8.25 aA	4.5 a A
	Concentrations			
	0.5 mg.L^{-1}	**1.0 mg.L^{-1}**	**1.5 mg.L^{-1}**	**2.0 mg.L^{-1}**
F K	10.0 aA	34.5 aAB	46.25 bB	92.25 b C
Absence of phosphite		102.0		
Imazalil		0.0		
CV (%)		75.73		

Means followed by the same lowercase letter in the column and upper case in the row do not differ by the Tukey's test (P <0.05). Means of treatments followed by the letter X and means of treatments followed by the letter Y differ statistically from controls without phosphite and Imazalil, by the Dunnet's test (P <0.05) respectively

FCu2 showed the greatest reduction in the production of *C. musae* conidia.

Spores are reproductive and infective units of phytopathogenic fungi responsible for producing propagules that spread and infect the plant. Thus, the greater the inhibition of spore formation, the more efficient is the product, and this is a very important feature in the pathogen management in the field [31,34].

In the comparison of treatments to controls, it was verified that only treatment using FK at concentration of 1.5 and 2.0 mL.L^{-1} did not reduce sporulation (Table 2). In the comparison with Imazalil, it was verified that FCu1 at concentration of 0.5 mL.L^{-1} and FK at concentrations of 1.5 and 2.0 mL.L^{-1} differed from this treatment, indicating that the fungicide was more effective in the control of conidia production than these treatments. There are reports that some products may serve as a stimulus for the fungus to reproduce [35].

When increasing the FCu1 concentration, sporulation control of up to 100% is verified, being similar to Imazalil fungicide. For FCu2, it was verified that from the lowest concentration applied, no significant difference from Imazalil was observed, demonstrating that FCu2 was as efficient as the fungicide in the control of this variable.

It is likely that sporulation inhibition occurs due to the fungistatic effect of higher phosphite concentrations on the mycelial growth, which

results in the change from vegetative to reproductive stages as a survival strategy of the microorganism [36].

The results observed for the FK treatment obtained in the present experiment differ from those obtained in studies using similar sources of phosphite in different phytopathogens [34, 29,31].

For germination, the results presented in Table 4 show that FCu1 yielded the highest *C. musae* germination reduction at concentrations of 1.5 and 2.0 $mL.L^{-1}$. For FCu2, the highest germination reduction was obtained at concentration of 1.0 $mL.L^{-1}$. FK showed no significant difference among applied concentrations. It is verified that only FCu1 at concentration of 1.5 $mL.L^{-1}$ did not differ from control using the fungicide, demonstrating the efficiency of this treatment.

By fixing the concentrations used in the different phosphate formulations, it is possible to verify that at concentrations of 0.5 and 1.0 $mL.L^{-1}$, FCu2 presented lower germinated conidia values. At concentration of 1.5 and 2.0 $mL.L^{-1}$, FCu1 promoted greater control in the germination of *C. musae* conidia.

At all concentrations tested, FCu2 showed difference in relation to control treatment without the use of phosphites. This result demonstrates the efficacy of FCu2 when compared to the absence of phosphite. Although the results were superior to those obtained by the control, they are still considered unsatisfactory when compared to chemical control.

Comparing the results obtained to controls, it was verified that only FCu1 at concentrations of 1.5 and 2.0 $mL.L^{-1}$ did not statistically differ from control using Imazalil, showing inhibition percentage up to 99.75% (Table 2). This result demonstrates that these treatments showed the same efficiency of the fungicide in the control of *C. musae* germination.

When compared to the absence of phosphite application, it was verified that FK did not differ significantly at any concentrations used, as did FCu1 at concentration of 0.5 $mL.L^{-1}$. These results demonstrate inefficiency in the control of *C. musae* germination.

Phosphites can act directly by inhibiting fungal spore germination, penetrating in the plant, blocking mycelial growth and spore production. Indirectly, they act by stimulating the metabolism involved in the resistance induced in the plant, as in the production of lignin, phytoalexin and hydrolytic enzymes [37].

Several studies using potassium phosphite report the efficiency of phytopathogen control, and these results are different from those obtained in this experiment. Tests carried out by Alexandre et al. [17], with K, Mg and Cu phosphites found that the germination of *Colletotrichum gloeosporioides* conidia was inhibited even at low concentrations (0.25; 0.5; 0.75 $g.L^{-1}$). Ribeiro Júnior, et al. [38] reported that even at reduced doses, potassium phosphite had toxic effect on the germination of *Verticillium dahliae* conidia. This trend was also verified by Ogoshi et al. [11], in the germination control of *Colletotrichum gloeosporioides* of up to 63.1% with the use of potassium phosphite at concentration of 10.0 $mL.L^{-1}$.

Table 4. Germination of *Colletotrichum musae* conidia under different sources of phosphite at different concentrations

	Concentrações			
Fontes de Fosfito	**0.5 $mL.L^{-1}$**	**1.0 $mL.L^{-1}$**	**1.5 $mL.L^{-1}$**	**2.0 $mL.L^{-1}$**
F Cu 1	89.25 bC	39.5 bB	3.25 aA	0.25 aA
F Cu 2	48.25 aB	22.0 aA	48.25 bB	37.0 bB
	Concentrações			
	0.5 $mg.L^{-1}$	**1.0 $mg.L^{-1}$**	**1.5 $mg.L^{-1}$**	**2.0 $mg.L^{-1}$**
F K	96.5 bA	90.5 cA	88.25 cA	91.0 cA
Ausência de fosfito			100.0	
Imazalil			0.0	
CV (%)			13.15	

Means followed by the same lowercase letter in the column and upper case in the row do not differ by the Tukey's test (P <0.05). Means of treatments followed by the letter X and means of treatments followed by the letter Y differ statistically from controls without phosphite and Imazalil, by the Dunnet's test (P <0.05) respectively

Phosphites have demonstrated fungal control potential, both in *in vitro* and *in vivo* conditions. The results obtained here encourage the conduction of further studies for the alternative management of banana anthracnose with the use of less toxic products.

4. CONCLUSION

Copper and potassium phosphites reduce the mycelial growth of *Colletotrichum musae* when compared to the absence of treatment.

FCu2 presents a fungicide-like effect from concentration of 0.5 $m.L^{-1}$ on the control of *C. musae* conidia production.

FCu1 presents a fungicide-like effect from concentration of 1.5 $mL.L^{-1}$ on the control of *C. musae* germination.

In view of the results obtained in the experiment, it is concluded that the application of phosphites is viable in the control of the development of *C. musae in vitro*.

ACKNOWLEDGEMENT

The authors thank Capes and Fapemig for the financial support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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