Original Research Article

Antifungal activity of copper, zinc and potassium compounds on mycelial growth and conidial germination of *Fusarium solani* f. sp. *piperis*

ABSTRACT (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

Fusariosis is a disease that causes economic damage to black pepper (*Piper nigrum* L.) producers. Despite being a major disease, there is no record of efficient chemical control. Thus, the objective was to evaluate the antifungal activity of copper, zinc and potassium compounds in mycelial growth and conidial germination of *Fusarium solani* f. sp. *piperis* in vitro. For inoculation in PDA (Potato Dextrose Agar) medium, 7 mm discs from the pure culture were transferred to Petri dishes. The plates were incubated at 25°C in a biochemical oxygen demand (BOD) chamber, with photoperiod of 12 h, for 15 days. Micronutrients were supplied as sulfates, $CuSO_4$ (copper sulfate) and $ZnSO_4$ (zinc sulfate), at concentrations of 1, 5, 10, 15 and 20 mmol/L. Potassium macronutrient (K) was supplied as KCl (potassium chloride) at concentrations of 30, 60, 90,120 and 150 mmol/L. The experiment was performed using a completely randomized design with 6 treatments and ten replications. $CuSO_4$ showed fungicidal effect at concentrations of 10, 15 and 20 mmol/L. For $ZnSO_4$ mycelial growth was completely inhibited at concentrations of 15 and 20 mmol/L. There was no inhibition or reduction of fungal growth in the presence of K. Copper and zinc at minimal concentrations were efficient in controlling mycelial growth and inhibition of spore germination of *F. solani* f. sp. *piperis*. In contrast, potassium did not exert fungicidal or fungistatic effect on the fungus.

Keywords: Fusarium solani, antigungal activity, fungal growth inhibition, nutrients

1. INTRODUCTION (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

Pathogenic fungi include a large and heterogeneous group of organisms that occupy an important position in both agriculture and natural populations [1]. The genus *Fusarium* spp. is classified as ascomycet, characterized by a hyaline mycelium, branched and septate, with phyllide-shaped sporophores and conidia of varying size and shape. It has spindle-shaped macroconids with several septa [2]. *Fusarium* species are known as phytopathogens, saprophytes with worldwide distribution [3].

Fusarium solani (Mart.) Appel & Wr. emend. Snyd. & Hans. f. sp. *piperis*, Albuquerque (Telemorph *Nectria haematococca* Berk. & Br. f. sp. *piperis* Albuq.) is the causal agent of fusariosis and has brought great economic damage to black pepper (*Piper nigrum* L.)

producers, decimating whole crops. The disease may start in the root system or in the shoot. When initiated by the roots, the root system is reduced and becomes necrotic, causing yellowish and flaccid leaves and premature fall. On the other hand, once started in the aerial part, it is characterized by the presence of yellowish branches in a very vigorous plant. With the evolution of the disease is observed drying in the upper and lower part of the plant [4-6]. According to Pandey et al. [7], production losses due to plant diseases are a considerable challenge for the current agricultural production system worldwide, representing at least 25% of the total. Although fusariosis is a major disease, there is no record of efficient chemical control.

The methods used in the control of pathogenic isolates of *Fusarium* include the use of resistant varieties and soil disinfection with the chemical fungicide and crop rotation using non-host plants [8]. The use of resistant cultivars would be an alternative, but difficulties such as the identification of resistance genes or the pathogen's ability to adapt to new genotypes may make resistance a temporary solution [9]. In addition, the chemical control of *Fusarium* spp. is not fully efficient since the pathogen penetrates the vascular tissue of the plant [10]. Crop rotation would be of little efficiency since this pathogen is a soil fungus, capable of surviving for long periods in crop debris and presents several plant species as hosts [9]. Conventional synthetic fungicides are largely considered the most effective and economical means for treating the disease. However, the intensity of use and specific mode of action of most synthetic fungicides ultimately lead to resistance problems and an increased environmental cost [7, 11, 12]. Thus, alternative forms of control are of increasing interest, leading to the investigation and development of effective and sustainable products for the control of plant pathogens [13]. Zambolim et al. [14] reported that some micro and macronutrients have been identified as one of the main mineral elements associated with the induction of disease resistance in plants.

Due to their low cost, protective activity and reduced risk of resistance development controlled by the broad mode of action against pathogens, Cu compounds have been exploited to protect crops from many pests, including those that cause numerous bacterial and fungal infections [15]. According to Zambolim et al. [16], Cu ions in contact with spores or the pathogen's germ tube may accumulate in the membrane or penetrate and concentrate inside of the spores or mycelium, where they act by inhibiting enzymes essential to the metabolic process of microorganisms. Once accumulated in the cells, their effects become irreversible.

Interest in research on zinc (Zn) derivatives is increasing [17, 18], due to its strong antimicrobial activity at low concentrations and its non-toxic characteristics in adequate quantities. Zn as an essential micronutrient plays an important role in many integral metabolic processes [19]. It can also help increase chlorophyll and carotenoid biosynthesis and improve plant photosynthetic apparatus [20]. Significant optoelectric, physical and antimicrobial properties of Zn offer great potential for increasing agricultural productivity [21]. Its mode of action is not completely understood, but it is known to act directly on the pathogen [16].

Among macronutrients of great importance to the plant, potassium (K) is one of the elements that has very positive results in reducing the incidence of pests and diseases [22, 23], being able to reduce the severity of more of 100 fungi [24]. Taiz and Zeiger [25] reported that K is an essential plant nutrient required as a cofactor for over 40 enzymes, many of which are involved in respiration and photosynthesis. As such, it is an important nutrient in plant disease prevention as it is involved in many cellular processes that influence disease severity. Its effect on the prevention of diseases caused by bacteria, fungi and nematodes has been reported [16, 26]. Increased resistance to disease from K fertilization has been

attributed to several mechanisms, such as cell permeability and decreased susceptibility of tissues to pathogen maceration and penetration [16, 27]. K influences the reduction of plant diseases due to the activation of enzymes involved in respiration and photosynthesis, carbon chain supply processes for defense substance synthesis, as well as stomatal regulation influencing mass flow solute transport [28].

The use of nutrients with antifungal action may be a strategy for controlling pathogens that cause invaluable economic losses. Nutrients such as Cu, Zn and K are easily accessible, inexpensive and still contribute to plant nutrition. Thus, the objective of this study was to evaluate the antifungal activity of Cu, Zn and K compounds against *Fusarium solani* f. sp. *piperis* growth.

2. MATERIAL AND METHODS

2.1. Microorganism and cultivation

The isolate of *Fusarium solani* f. sp. *piperis* CML 2466, from the Coleção Micológica de Lavras, Federal University of Lavras - MG was used. The fungus was maintained on Petri dishes containing PDA (Potato Dextrose Agar) at 4 ° C. For inoculation, 7 mm pure culture discs were transferred to Petri dishes containing the same medium. The plates were incubated at 25° C in BOD (Biochemical Oxygen Demand), photoperiod of 12h for 15 days.

2.2. Copper, zinc and potassium concentrations

Micronutrients were supplied in PDA medium as copper sulfate ($CuSO_4$) and zinc sulfate ($ZnSO_4$) at concentrations of 1, 5, 10, 15 and 20 mmol/L. Potassium macronutrient (K) was supplied as potassium chloride (KCl) at concentrations of 30, 60, 90,120 and 150 mmol/L. The nutrients were diluted in sterile distilled water and at the time of plating were added to the PDA culture medium in laminar flow hood. After solidification, a 7 mm diameter fungal mycelium disc with 15-day-old was transferred to the center of each petri dish (68 mm diameter). The PDA medium with the fungus disc was used as control. The plates were sealed with Parafilm and maintained in BOD at 25ºC with 12 h photoperiod.

2.3. Mycelial growth

The evaluation of *F. solani* mycelial growth in the control plates and treatments was determined every 2 days by measuring the diameter of the colonies in orthogonal directions with the digital pachymeter until the control treatment colony reached edge of the plate, ie 12 days after inoculation (DAI). The growth inhibition percentage was calculated according to Guo et al. [29], where the antifungal index (%) = (1-Da / Db) x 100, where: Da gives the diameter of the growth zone in the test plate and Db the diameter of the growth zone in the control plate.

2.4. Spore count

The spore suspension was prepared by adding 20 mL of sterile distilled water to each plate containing the fungus, which was scraped with a Drigalsky handle for efficient spore extraction. The spore count was performed in a Neubauer Chamber and the suspension was adjusted to a concentration of 106 spores/mL^{-1}.

2.5. Statistical analysis

Comment [LL1]: Actually you have 3 experiments (Cu, Zn and K) with 5 doses for each and analyzed in 6 periods (2, 4, 6, 8, 10 and 12 days). You should improve your statistics. Quantitative data is performed regression and not average test.

The experiment was performed using a completely randomized design with 6 treatments and 10 replications for each treatment (Cu, Zn and K). Each repetition consisted of a petri dish. All data were submitted to analysis of variance (ANOVA) and Tukey test using Genes software [30].

3. RESULTS

3.1. Mycelial growth

The mycelial growth of *F. solani* was dependent on the nutrient and the dose used. Fungal growth was completely inhibited in some treatments. The $CuSO_4$ showed fungicidal effect at concentrations of 10, 15 and 20 mmol/L, completely inhibiting growth of the colonies (Fig. 1a). However, at a concentration of 5 mmol/L, a fungistatic effect was observed since mycelial growth was initiated 6 DAI (Fig. 2a). For $ZnSO_4$ mycelial growth was completely inhibited at concentrations of 15 and 20 mmol/L, showing fungicidal effect and significantly reduced ($P \leq 0.05$) at concentrations of 5 and 10 mmol/L, exerting fungistatic effect (Fig. 1b). This result is ratified after eight days of incubation by observing mycelial growth at a concentration of 10 mmol/L (Fig. 2b). There was a significant difference for KCl treatment ($P \leq 0.05$) between the tested concentrations. However, there was no inhibition or reduction of fungal growth in the presence of this nutrient (Fig. 1c and 2c).

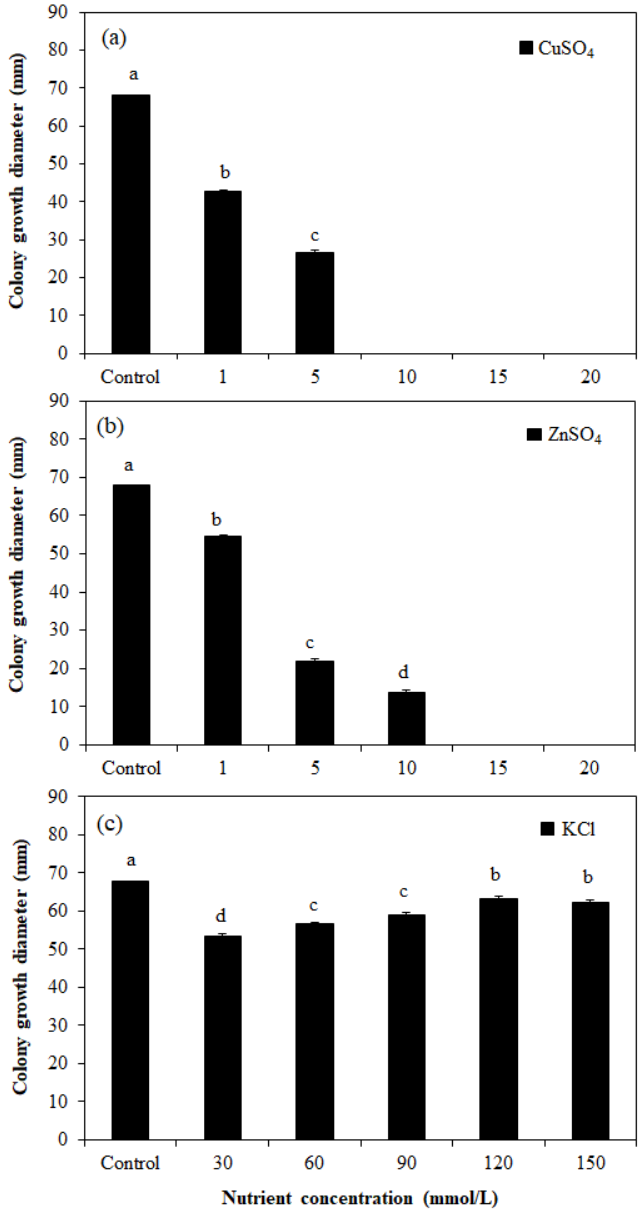

Fig. 1. Antifungal activities of $CuSO_4$ (a), $ZnSO_4$ (b) and KCl (c) against *F. solani* f. sp. *piperis* on PDA at different concentrations.

Data are shown as average values. Columns followed by the identical letter are not statistically different according to by Tukey test, at 5% probability (P≤0.05). Bars represent the standard error of the mean.

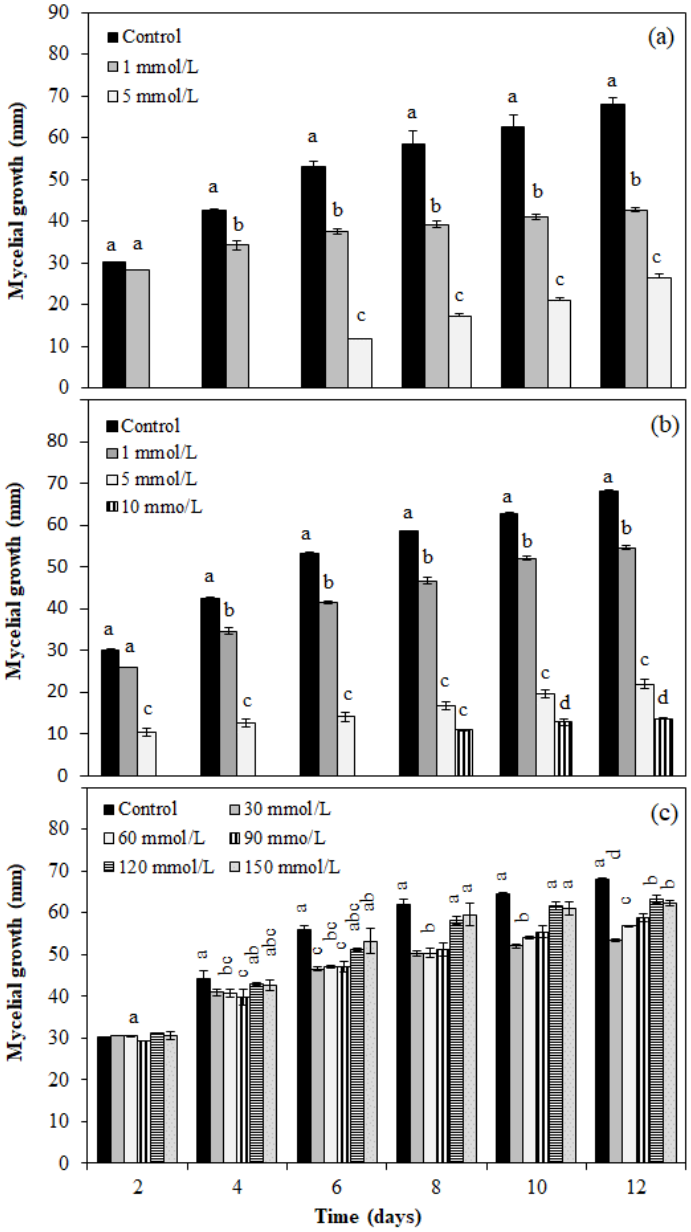

Fig. 2. Effect of $CuSO_4$ (a), $ZnSO_4$ (b) and KCl (c) on the mycelial growth of *F. solani* f. sp. *piperis*, 12 days after inoculation. Control: only PDA medium.

Data are shown as average values. Columns followed by the identical letter are not statistically different according to by Tukey test, at 5% probability (P≤0.05). Bars represent the standard error of the mean.

3.2. Percent growth inhibition (P.I.).

The mycelial growth inhibition index confirmed the efficiency of the antifungal activity of $CuSO_4$ and $ZnSO_4$ (Table 1). For $CuSO_4$, at a concentration of 5 mmol/L, there was inhibition greater than 50% 12 DAI. The other concentrations inhibited 100% fungal growth. Similar results were observed for $ZnSO_4$ (Table 1). However, for KCl, in none of the evaluated concentrations was observed P.I. below 50%. At 2 DAI there was growth induction (Table 1) with no fungistatic or fungicidal effect for this nutrient.

Table 1. Percent growth inhibition (P.I.) of *F. solani* f. sp. *piperis* in $CuSO_4$, $ZnSO_4$ and KCl

	P.I. (%)					
Time (days)	**2**	**4**	**6**	**8**	**10**	**12**
$CuSO_4$ mmol/L						
Control	0	0	0	0	0	0
1	12.0	19.4	29.4	33.0	34.5	37.2
5	100	100	77.8	70.7	66.4	61.0
10	100	100	100	100	100	100
15	100	100	100	100	100	100
20	100	100	100	100	100	100
$ZnSO_4$ mmol/L						
Control	0	0	0	0	0	0
1	14.4	18.8	22.0	20.3	17.0	19.8
5	65.7	70.4	73.6	71.5	68.9	67.7
10	100	100	89.9	81.5	79.5	79.9
15	100	100	100	100	100	100
20	100	100	100	100	100	100
KCl mmol/L						
Control	0	0	0	0	0	0
30	-0.9	7.1	16.6	18.7	19.2	21.4
60	-3.8	8.0	15.6	18.8	16.2	16.5
90	2.8	9.8	15.8	17.4	14.0	13.3
120	-3.0	2.7	8.2	6.1	4.4	7.0
150	-1.4	3.7	4.8	3.8	5.0	8.3

3.3. Conidia Number.

Twelve days after inoculation (12 DAI), the conidia number of *F. solani* was inhibited in the presence of $CuSO_4$, $ZnSO_4$ and KCl. The Cu micronutrient reduced by 84% the conidial germination at 1mmol/L concentration in relation to the control. The same was not observed for Zn at the same concentration (Figure 3a, Table 2). In the other Cu and Zn concentrations, conidial germination was significantly inhibited (*P≤0.05*), with values greater

than 80%. For K treatment, there was a 20.6% reduction in the number of conidia at 30 mmol/L. The other concentrations presented a reduction greater than 50% when compared to the control (Figure 3b, Table 2).

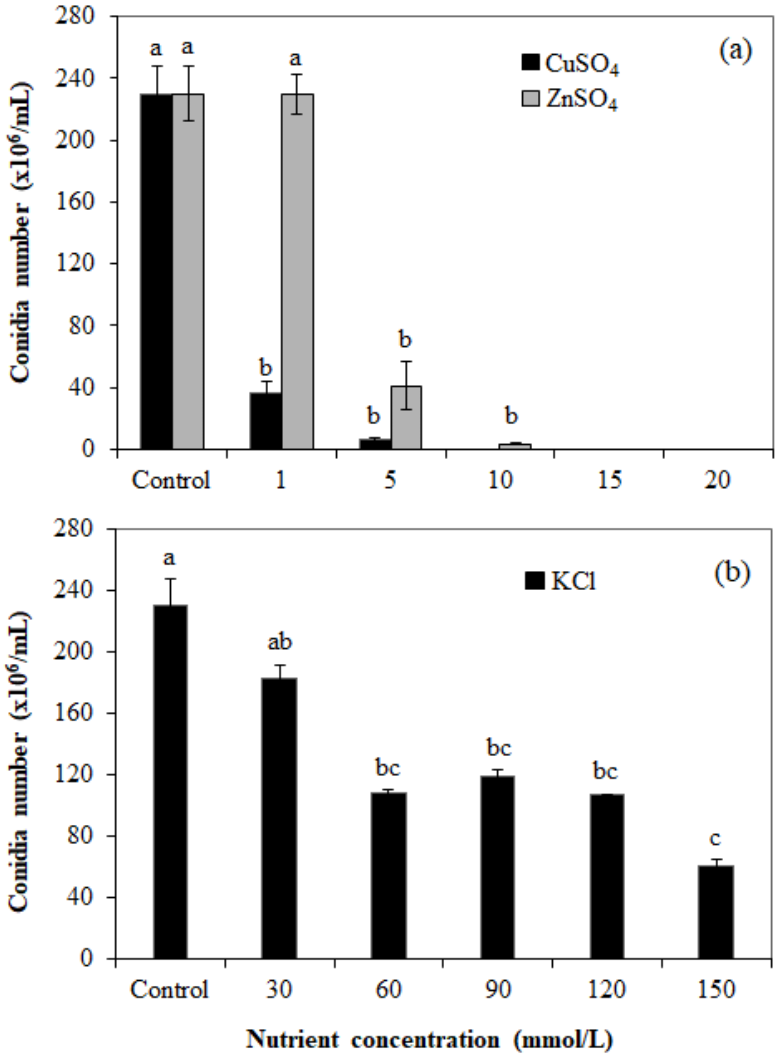

Fig. 3. Conidia production of *F. solani* f. sp. *piperis* on $CuSO_4$, $ZnSO_4$ (a) and KCl (b) in different concentrations.

Data are shown as average values. Columns followed by the identical letter are not statistically different according to by Tukey test, at 5% probability (P≤0.05). Bars represent the standard error of the mean.

Table 2. Percent growth inhibition (P.I.) of *F. solani* f. sp. *piperis* in $CuSO_4$, $ZnSO_4$ and KCl

Reduction in Conidia number (%)	
mmol/L	$CuSO_4$
1	84.2
5	97.2
10	100.0
15	100.0
20	100.0
	$ZnSO_4$
1	0.0
5	82.2
10	98.4
15	100.0
20	100.0
	KCl
30	20.6
60	52.9
90	48.3
120	53.6
150	73.5

4. DISCUSSION

The widespread and persistent nature of *Fusarium* spp. may be due to its ability to maintain and multiply in a wide variety of complex carbohydrates and proteins, thus resisting adverse climates and high levels of toxic substances such as many antibiotics and fungicides. *F. solani* seems to incorporate some of the most difficult members of the genus [31].

Inhibition of mycelial growth of *F. solani* f. sp. *piperis* in vitro revealed significant variations in fungus sensitivity to different nutrients tested. In the present study, $CuSO_4$, $ZnSO_4$ were more effective in inhibiting fungal growth, while KCl showed relatively weak effect comparing it to other nutrients. However, it was observed that Cu was the most effective compound against mycelial growth and *F. solani* conidia germination, showing significant inhibition at relatively low concentrations of the compound.

According to Resende et al. and Melo et al. [32, 33], Cu, Zn and K compounds are used for the control of microorganisms. They can have a direct effect on the pathogen (fungicidal or fungistatic effect) or activate the natural defense of plants, resulting in induced resistance [34, 35].

The Cu is currently used due to its antifungal properties. In particular, Cu is responsible for interference with homeostatic processes and cell membrane functions, protein synthesis damage, reactive oxygen species production, and DNA disruption [36, 37]. Civardi et al. [38] observed that Cu exerted toxic effect on *Rhodonia placenta* fungal cell by breakdown of different basic metabolic processes. Significant antifungal activity of Cu has been revealed in

a number of pathogenic species including *Fusarium* sp., *Aspergillus niger*, *Rhizoctonia solani*, *Alternaria solani*, *Alternaria alternate* and *Phoma destructiva* [7, 39, 40].

Regarding Zn, several studies have shown its antibacterial activity [41-44]. However, there are few studies reporting the suggested mechanism for antifungal activity of Zn compounds [45, 46]. Some authors suggest that such a mechanism may be based on the formation of reactive oxygen species that disrupt the integrity of the cell membrane, preventing pathogen growth [47, 48, 44, 46]. According to He et al., Król et al. and Ashajyothi et al. [46, 49, 50], Zn compounds showed fungistatic potential against *Fusarium* sp., *Botrytis cinerea*, *Penicillium expansum, Aspergillus niger* and *Rhizopus stolonifer*. Chand et al. [51] observed that among the micronutrients tested Zn presented the greatest inhibition of mycelial growth of *Fusarium oxysporum* f. sp. *cuban*.

The marked toxic effect of Cu and Zn against fungal spores compared to mycelial growth can be attributed to the structural differences between the spore wall and the fungal vegetative phase. Bartnicki-Garcia [52] observed that the chitin content of many fungal species is significantly higher in the hyphae wall compared to the spore wall, making the latter more susceptible to some compounds. In addition, during the spore germination process, the presence of enzymes such as disulfide reductase and glucanases result in weakening of the cell wall, facilitating germ tube lengthening and thus creating sites of greater sensitivity to toxic substances in contact with the cell fungal. In general, conidial germination may reflect reproductive capacity and fungal development. Savi et al. [18] suggest that the effect of Zn compounds on fungal growth may be related to their property, altering reproductive capacity in terms of conidia viability. Malandrakis et al. [53], studying the effect of copper and zinc on various microorganisms, found that Cu was effective against *Alternaria alternata*, *Botrytis cinerea*, *Monilia fructicola*, *Verticillium dahliae*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum* f. sp. *radicis lycopersici* and *Fusarium solani* while Zn exerted a fungicidal effect against *M. fructicola*, *F. solani* and *V. dahliae*.

Although K has not exerted inhibition or reduction of *F. solani* mycelial growth and spore production, there are reports in the literature that K acts as an inducer of resistance to plant diseases [54-56]. The use of K as a plant fertilizer may decrease the incidence of fungal and bacterial as well as insect diseases [57], mainly due to changes in primary metabolism and plant hormonal responses [27, 55]. Dordas [58] observed that the application of KCl on foliage can prevent the attack of mildew on wheat.

5. CONCLUSION

The results obtained in this work provide evidence that copper and zinc exhibited beneficial antifungal activity against *F. solani* f. sp. *piperis* under laboratory conditions. In contrast, no potassium concentration was active against the fungus. High antifungal activity was observed at low concentrations of copper and zinc, favoring the use of these compounds. Scientific evaluations are being carried out in the field to verify the performance of these nutrients as growth inhibitors of *Fusarium*.

COMPETING INTERESTS

The authors declare that they have no conflicts of interest.

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DEFINITIONS, ACRONYMS, ABBREVIATIONS

Here is the Definitions section. This is an optional section.

Term: Definition for the term