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Germination of fungal pathogen spores in calcium, copper, manganese and zinc chelated with aminoacids[1]

Germinação de esporos fúngicos em quelatos à base de cálcio, cobre, manganês e zinco

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ABSTRACT - Due to the environmental impact generated using fungicides, new alternatives for disease control have been arising, such as the use of nutrients that may act on the development of certain pathogens or decrease the host susceptibility to diseases. The objective of this work was to verify if chelates of calcium, copper, manganese and zinc inhibit the germination of *Puccinia sorghi*, *Cercospora* sp. and *Exserohilum turcicum* spores. Two experiments were carried out: the first with agar-water culture medium, where the treatments and spores were placed at the same moment and the second using corn leaves as substrate, where treatments were administered zero, one, two, three, four and five hours after the spores are sprayed. Calcium, copper, manganese and zinc products were used as treatments in the form of chelate 15%, 5%, 15% and 10%, fungicide (strobilurine and triazole, 20% azoxistrobin and 8% ciproconazole + triazole, 25% propiconazole) and distilled water. The spores were counted 24 hours after the application of the treatments. In the first experiment, chelates showed an average reduction of 39% in both germination of *P. sorghi* and *Cercospora* sp. spores. For the second assay, the same pathogens had a mean reduction of 52%, 59%, 77%, 87%, 81% and 64% for *P. sorghi* and 40%, 33%, 17%, 18%, 4% and 2% for *Cercospora* sp., both at zero, 1, 2, 3, 4 and 5 hours after treatment, respectively. *E. turcicum* had no inhibitory effect under treatments. The metal-chelates tested were fungitoxic to *P. sorghi* and *Cercospora* sp. spores.

Key words: Alternative control. Nutrients. Chelates. *Zea mays* L..

RESUMO - Devido ao impacto ambiental gerado pelo uso indiscriminado de fungicidas, busca-se novas alternativas para o controle das doenças, como a utilização de nutrientes, os quais podem inibir o desenvolvimento de patógenos ou diminuir a suscetibilidade da planta a doenças. O objetivo deste trabalho foi verificar se produtos à base de cálcio, cobre, manganês e zinco inibem a germinação de esporos de *Puccinia sorghi*, *Cercospora* sp. e *Exserohilum turcicum*. Foram realizados dois experimentos: o primeiro com meio de cultura ágar-água, onde os tratamentos e os esporos foram colocados no mesmo momento; e o segundo utilizando folhas de milho como substrato, onde os tratamentos foram administrados zero, uma, duas, três, quatro e cinco horas após os esporos serem pulverizados. Utilizou-se como tratamentos produtos à base de cálcio, cobre, manganês e zinco na forma de quelato 15%, 5%, 15% and 10%, fungicida (estrobilurina e triazol, 20% azoxistrobin and 8% ciproconazole + triazol, 25% propiconazole) e água destilada. Realizou-se a contagem dos esporos 24 horas após a aplicação dos tratamentos. Para o primeiro ensaio, os quelatos apresentaram, em média, redução de 39%, tanto na germinação de esporos de *P. sorghi* quanto de *Cercospora* sp.. Para o segundo experimento, os mesmos patógenos tiveram redução média de 52%, 59%, 77%, 87%, 81% e 64% para *P. sorghi* e 40%, 33%, 17%, 18%, 4% e 2% para *Cercospora* sp., ambos nos tempos, zero, 1, 2, 3, 4 e 5 horas após o tratamento, respectivamente. Para *E. turcicum* não houve efeito inibitório em nenhum ensaio. Conclui-se que os metais na forma de quelato testados apresentam atividade antifúngica a *P. sorghi* e *Cercospora* sp..

Palavras-chave: Controle alternativo. Nutrientes. Quelatos. *Zea mays* L..

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INTRODUCTION

Corn (*Zea mays* L.) is one of the most cultivated cereals in the world (GALVÃO *et al*., 2014). The increment in the production of this crop is due to anticipation of sowing and increase of the second crop cultivation, which also contributes to the emergence of new pathogens (COSTA *et al*., 2010) that limit productivity (LANZA *et al*., 2016). Diseases management is essential to achieve high incomes and greater profitability (ROSA *et al*., 2017).

Some diseases like rust and leaf spots are very frequent in corn (TROJAN; PRIA, 2018). Among the rust, the common rust (*Puccinia sorghi* Schwein) is the most important in the southern region of Brazil, being favored by high relative humidity and low temperatures. The symptoms of this disease are the appearance of elliptical and elongated pustules, which become brownish-black due to the development of teliospore. This pathogen also affects the clover of the genus *Oxalis*, increasing the inoculum for the maize crop (DUDIENAS *et al*., 2013).

Gray leaf spot is one of the most important diseases of maize, being caused by the species *Cercospora zea-maydis* Tehon & Daniels, *C. zeina* Crous & U. Braun and *C. sorghi* f. sp. *maydis* Ellis & Everht. Symptoms are olive to necrotic lesions, limited by ribbed and with rectangular shape. The ideal conditions for the development of the disease are high humidity and temperature between 22 e 30 ºC (CARVALHO; PEREIRA; CAMARGO, 2016; ENGELSING *et al*., 2011). Another important leaf disease is the Northern corn leaf spot caused by *Exserohilum turcicum* (Pass.) K. J. Leonard & E. G. Suggs, mainly at high humidity and temperature between 18 and 27 ºC, which symptons are elliptical and necrotic lesions (DE ROSSI; REIS; BRUSTOLIN, 2015).

The occurrence of these and other diseases in maize crops demand greater use of fungicides (SCHUMACHER *et al*., 2017), which may increase environmental pollution and cause damage to human and animal health when used in an inappropriate manner.

Maize is a nutrient-demanding crop (OLIVEIRA *et al*., 2013) and nutritional balance is considered an important factor in the control of diseases (MORALES; SANTOS; TOMAZELI, 2012). Products based on calcium, copper, manganese, and zinc can contribute to physiological processes in the plant, improving their resistance to the pathogens, as well as may have direct toxic action on the phytopathogens.

Thus, the objective of this work was to verify whether products based on calcium, copper, manganese, and zinc can inhibit the germination of spores of the causal agents of common rust, gray leaf spot, and Northern corn leaf spot, in culture medium and in maize leaves at different times of application.

MATERIAL AND METHODS

Two assays were carried out. The first to verify whether the products based on calcium, copper, manganese, and zinc affect the germination of spores when applied directly on agar-water culture medium, and the second to find the time of absorption of the products and their action on the corn leaf inoculated with the pathogens.

In order to obtain spore suspension for both rust assays, uredospores of *Puccinia sorghi* were collected from clover leaves and placed in sterile distilled water containing 0.6% Tween 80. For gray leaf spot and Northern corn leaf spot, the pathogens were isolated from naturally infected maize leaves collected in the city of Toledo, Paraná, Brazil, and kept in potato-dextrose-agar (PDA) culture medium for *Cercospora* sp. and sorghum seed medium for *E. turcicum*. The spore suspension for both pathogens was obtained by washing fungal coloniewith sterile distilled water containing 0.6% Tween 80. Using Neubauer chamber, the number of spores was determined, and the suspension was calibrated to reach a concentration of $7x10^4$ spores mL^{-1} aproximately for the three pathogens.

Calcium, copper, manganese, and zinc-based products containing 15%, 5%, 15% and 10% of the nutrient in the form of amino acid chelate, respectively, were used in the doses recommended by the manufacturers. The fungicide 20% azoxistrobin and 8% ciproconazole at 3.0^{-6} mL cm^2 + 25% propiconazole at 4.0^{-6} mL cm^2 were used as control treatment.

In order to perform the *in vitro* assay, polystyrene box containing three sheets of moistened germ test paper were used, and on the paper, two sterilized wood sticks were put so that they fit inside the polystyrene box (0.1 m). On the sticks, four microscope slides were accommodated with a thin layer of about 1 mL 1% water-agar. One hundred µL of the treatments were placed onto water-agar culture medium and then 40 µL of spore suspension was deposited.

For the *in vivo* assay, the same structure was also used, however, the microscopy slides received a piece of corn leaf of approximately 0.0004 m^2. The leaves were collected in the field when the plants were in the phenological stage R_1, and all were free from any injury. The treatments were placed on the corn leaves using sprays, in the amount of 0.1 mL per leaf piece, to simulate the field application in adaxial surface. On each leaf piece, 40 µL of spore suspension was deposited at zero, 1, 2, 3, 4 and 5 hours after treatments.

For both *in vitro* and *in vivo* assays, the polystyrene box remained in the dark at 25 ºC for 24 hours and then the germinated spores were counted. The spore that had the germinative tube length greater than twice the diameter of the spore was considered germinated. The percentage of germinated spores was determined under a microscope by randomly counting the first 100 spores.

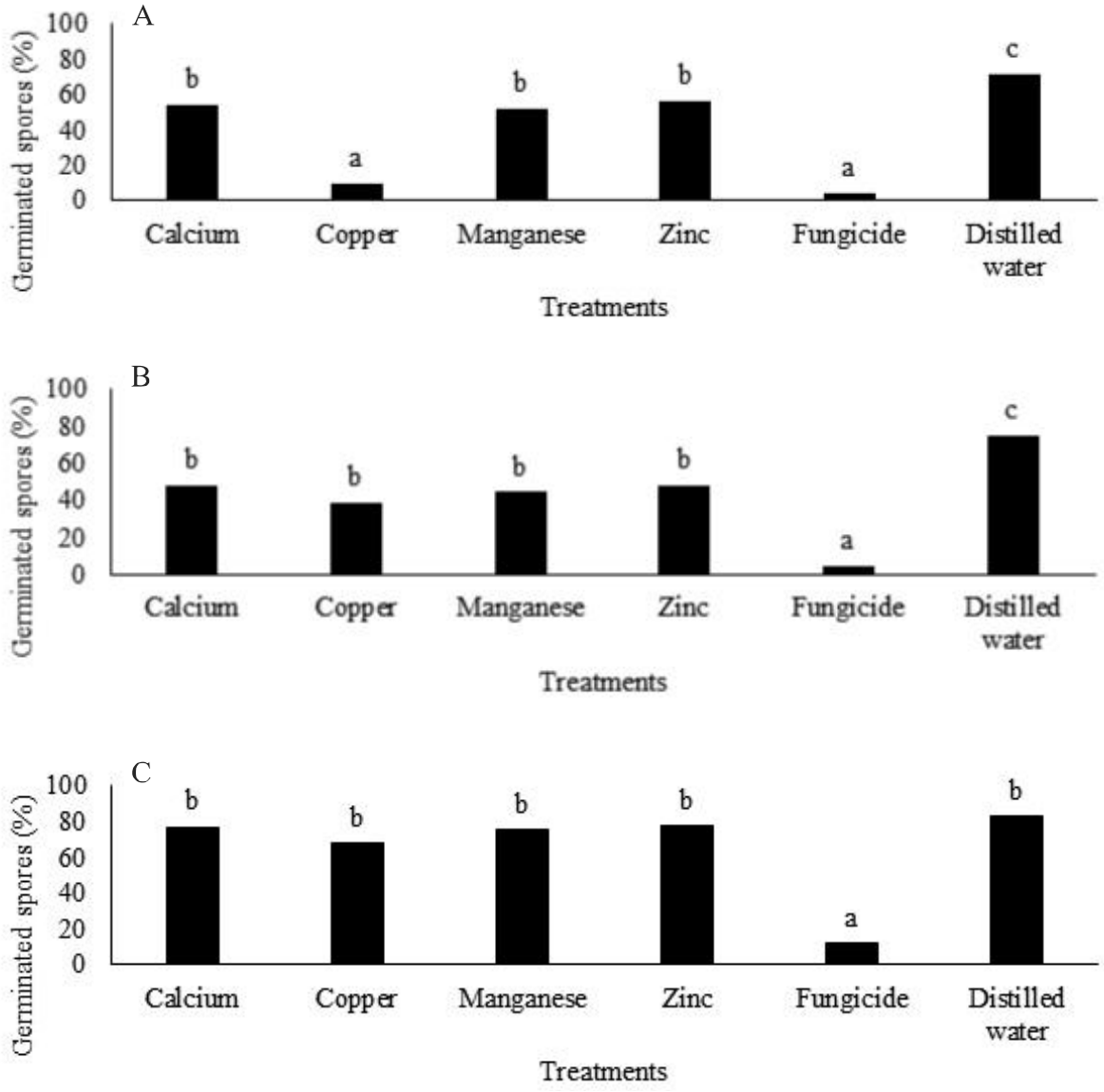
A completely randomized design was used, with four replications. Data were submitted to variance analysis and Tukey averages test at 5% probability through free software GENES (CRUZ, 2016).

RESULT AND DISCUSSION

For the *in vitro* assay using water-agar as a surface for the uredospores of *P. sorghi*, fungicide and copper-chelate treatments showed higher inhibition of spore germination, both differing from the treatments with calcium, manganese, zinc, and water. Spores treated with calcium, manganese, and zinc showed lower germination than water and higher than copper and fungicide. The water control treatment presented a higher number of germinated spores, differing from all other treatments (Figure 1A).

For *Cercospora* sp., the highest and lowest percentage of *in vitro* conidia germination occurred in the water and fungicide treatments respectively, both differing from all other treatments. Calcium, copper, manganese, and zinc-aminoacids based products not differed among them, with intermediate inhibition values between fungicide and water (Figure 1B).

Figure 1 - Germination of *Puccinia sorghi* (A), *Cercospora* sp. (B) e *Exserohilum turcicum* (C), spores on water-agar culture medium containing calcium, copper, manganese and zinc -aminoacids based products. Columns followed by the same letter do not differ statistically from each other by the Tukey ($p \leq 0.05$). CV (%): 11.33 (A), 17 (B), 10.12 (C)



These results demonstrate the efficacy of nutrients-aminoacids based products in decreasing the germination of *Cercospora* sp. and *P. sorghi* spores, however, without the same inhibitory effect presented by copper against the pathogen of common rust.

For *E. turcicum*, only the fungicide inhibited *in vitro* the spore germination (Figure 1C). There was no decrease in spore germination with the use of calcium, copper, manganese, and zinc-aminoacids based products, which confirms the hypothesis that products behave differently depending on the pathogen.

Concerning the *in vivo* assays, where corn leaf was used and pathogen inoculation was performed at different hours after treatment, for *P. sorghi*, when the treatment and uredospores were applied at the same time, fungicide presented the lowest percentage of germinated spores, differing from treatments with copper, manganese, zinc and water (Figure 2A), but not differing from the treatment with calcium. At the time 1 hour, the fungicide presented the highest inhibition, differing from the calcium, copper, zinc, and water, but not differing from the manganese. Treatments with calcium, copper, manganese, and zinc showed lower germination values than water (Figure 2B). When uredospores were added 2 hours after treatments (Figure 2C), the lowest percentage of germination was verified for the treatment with fungicide, manganese and copper that differed from zinc and water treatmens. For the inoculation 3 hours after treatment (Figure 2D) and 4 hours after treatment (Figure 2E), there was no difference between calcium, copper, manganese, zinc, and fungicide treatments. When the spores were placed 5 hours after the treatments, fungicide presented the highest inhibition for uredospore germination, but similar to manganese treatment (Figure 2F). At all times evaluated, the control treatment with water differed from the other treatments, always presenting the highest percentage of *P. sorghi* uredospores germination.

Based on the time between treatments and the arrival of the uredospores of *P. sorghi* it can be seen that for the treatments with calcium, copper, manganese and zinc, there was a decrease more pronounced in uredospore germination when they were added 2 and 3 hours after treatment. These lower values of germinated uredospores maintained in times 3 hours and 4 hours. From time 4 hours, except for manganese treatment, the others tended to lose their effectiveness due to increased spores germinated in the 5 hours time. Fungicide treatment maintained its basal levels of spore germination during all times evaluated, presenting constant toxic effect.

A possible explanation for these results would be that in the leaf microenvironment, the variation in the percentage of germinated uredospores may have occurred because the nutrients-based products activated the production and release of some compound by the plant, which would have acted on the spore germination since they can be easily absorbed by the cells acting inside the leaf. It may also be that a certain amount of products remain on the leaf surface after treatment which contributed to the decrease in the number of germinated spores by direct fungitoxic effect, as demonstrated in the *in vitro* water-agar assay.

In the *in vitro* assay trial, copper presented the greatest toxic effect to the spores of *P. sorghi*. In the soil, it has been showed that copper can cause deleterious effects in various life forms, as it alters the activity of enzymes, such as those involved in the respiratory process, affecting in a nonspecific way the diversity of microorganisms (SIQUEIRA; POUYU; MOREIRA, 1999).

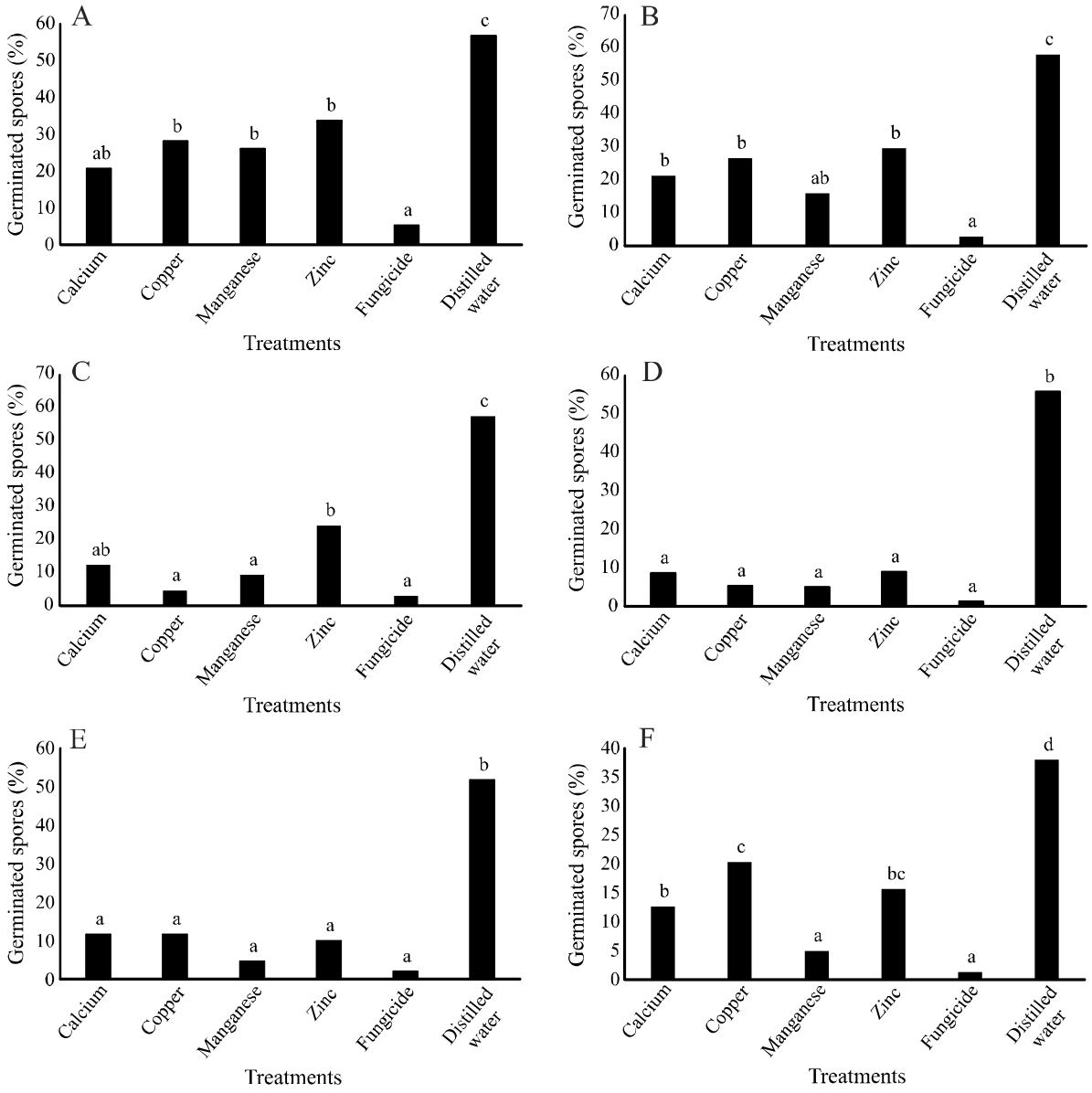
Copper improves lignification of cellular wall and in addition, Malavolta, Vitti and Oliveira (1997) reported the possible action of this element in the process of defense of plants, by inducing resistance. Concerning direct action on the pathogen, copper is readily accumulated by the fungal cell and forms complex with enzymes that have sulfidril groups, hydroxyl, amino or carboxyla, thus disabling them and disorganizing metabolism and integrity of spore or hypha (REIS; REIS; CARMONA, 2010).

According to Yamamoto *et al*. (2011), calcium is essential for the stability of biomembranas, and according to Morales, Santos and Tomazeli (2012) micronutrients such as copper and manganese are important because they influence the synthesis of lignin and phenols.

Calcium can act on enzymes produced by pathogens, especially pectolytics, such as polygalacturonases, which are used at the time of penetration and colonization (MORALES; SANTOS; TOMAZELI, 2012). In addition, calcium plays a key role in the structuring and biochemical composition of the plant cell, especially the cell wall and plasma membrane (WHITE; BROADLEY, 2003), accentuating host resistance to pathogen. Additionally, calcium ions can directly inhibit fungal growth, reduce ethylene production, and act as a signal for defense mechanisms related to variations in calcium levels in plant cells, or indirectly due the formation of the calcium-calmodulin complex, responsible for modulating physiological processes of plant response to biotic stresses (BEDENDO; AMORIM; MATTOS-JÚNIOR, 2018).

Manganese may also be related to resistance induction, activating various enzymes of the shikimic acid pathway and subsequent pathways, leading to biosynthesis of aromatic amino acids and secondary products such as lignin and flavonoids (CARVALHO *et al*., 2015). For zinc, there is evidence that it maintains the integrity of the

Figure 2 - Germination of *Puccinia sorghi* uredospores on corn leaf treated with calcium, copper, manganese, and zinc-aminoacids based products. Inoculation of the pathogen occurred at the same time as treatment (time zero hours) (A), and 1 (B), 2 (C), 3 (D), 4 (E) or 5 hours (F) after treatments. Columns followed by the same letter do not differ statistically from each other by the Tukey ($p \leq 0.05$). CV (%): 25.61 (A), 19.00 (B), 26.86 (C), 23.66 (D), 25.17 (E) e 14.76 (F)

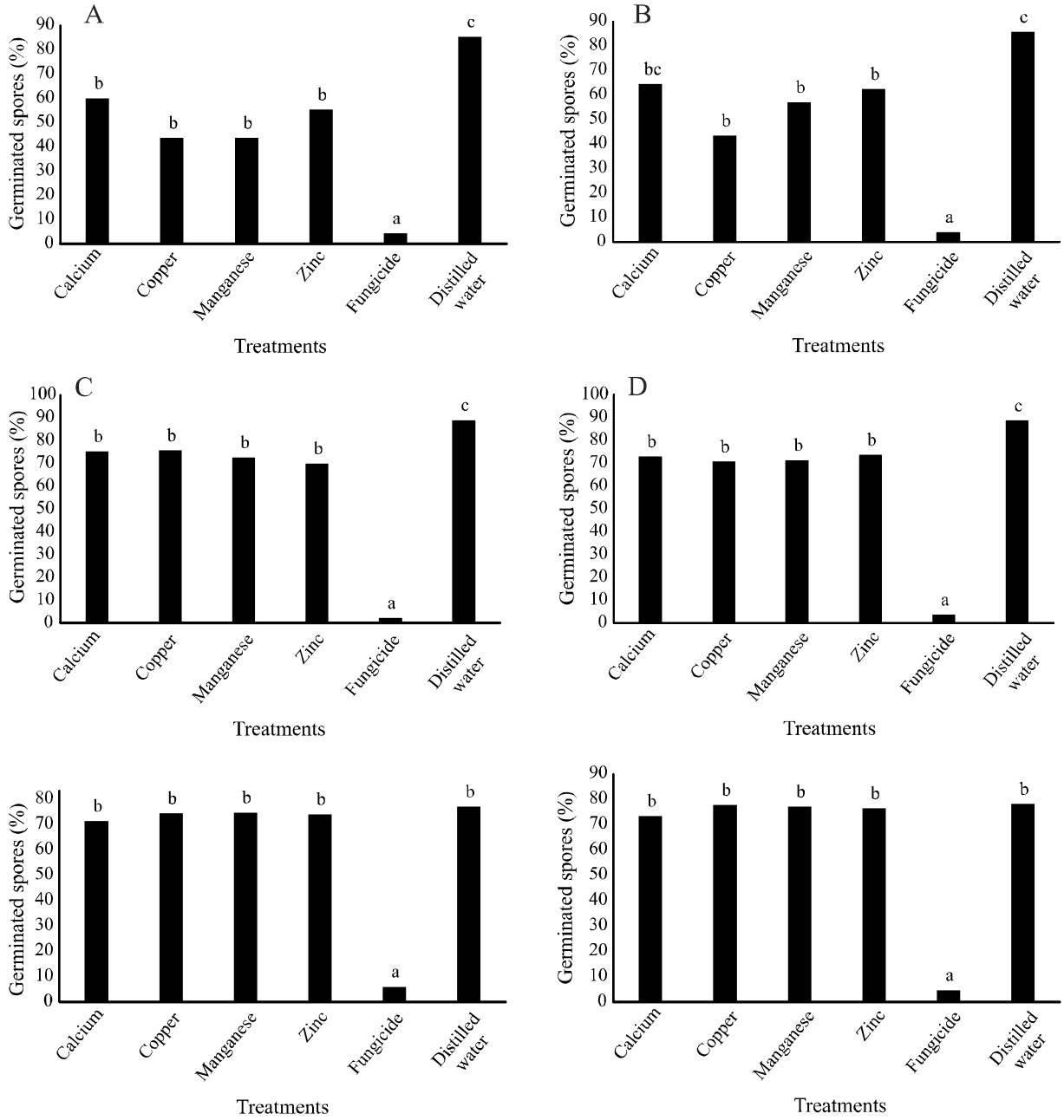


membrane (OHSE *et al*., 2012), having therefore action on the plant defense mechanisms.

In the *in vivo* assay with *Cercospora* sp. conidia, at time zero, fungicide presented the lowest percentage of germinated spores, differing from all other treatments. Treatments based on calcium, copper, manganese, and zinc were similar, but differed from water and fungicide (Figure 3A). When spores were inoculated 1 hour after treatments, the fungicide presented inhibition, differing from all other treatments. Copper, manganese and zinc differed from both water and fungicide, presenting intermediate values of spore germination (Figure 3B).

According to Figures 3C and 3D, in times 2 and 3 hours, it can be verified that again the treatments calcium, copper, manganese, and zinc presented intermediate spore germination values, as occurred in time zero, where they differed statistically from both water and fungicide. However, in times 4 and 5 hours after treatments (Figures 3E and 3F, respectively), only fungicide inhibited the germination of *Cercospora* sp. conidia.

Figure 3 - Germination of *Cercospora* sp. conidia on corn leaf treated with calcium, copper, manganese and zinc -aminoacids based products. Inoculation of the pathogen occurred at the same time as treatment (time zero hours) (A), and 1 (B), 2 (C), 3 (D), 4 (E) or 5 hours (F) after treatments. Columns followed by the same letter do not differ statistically from each other by the Tukey ($p \leq 0.05$). CV (%): 15.40 (A), 14.89 (B), 5.35 (C), 7.23 (D), 8.49 (E) e 5.99 (F)



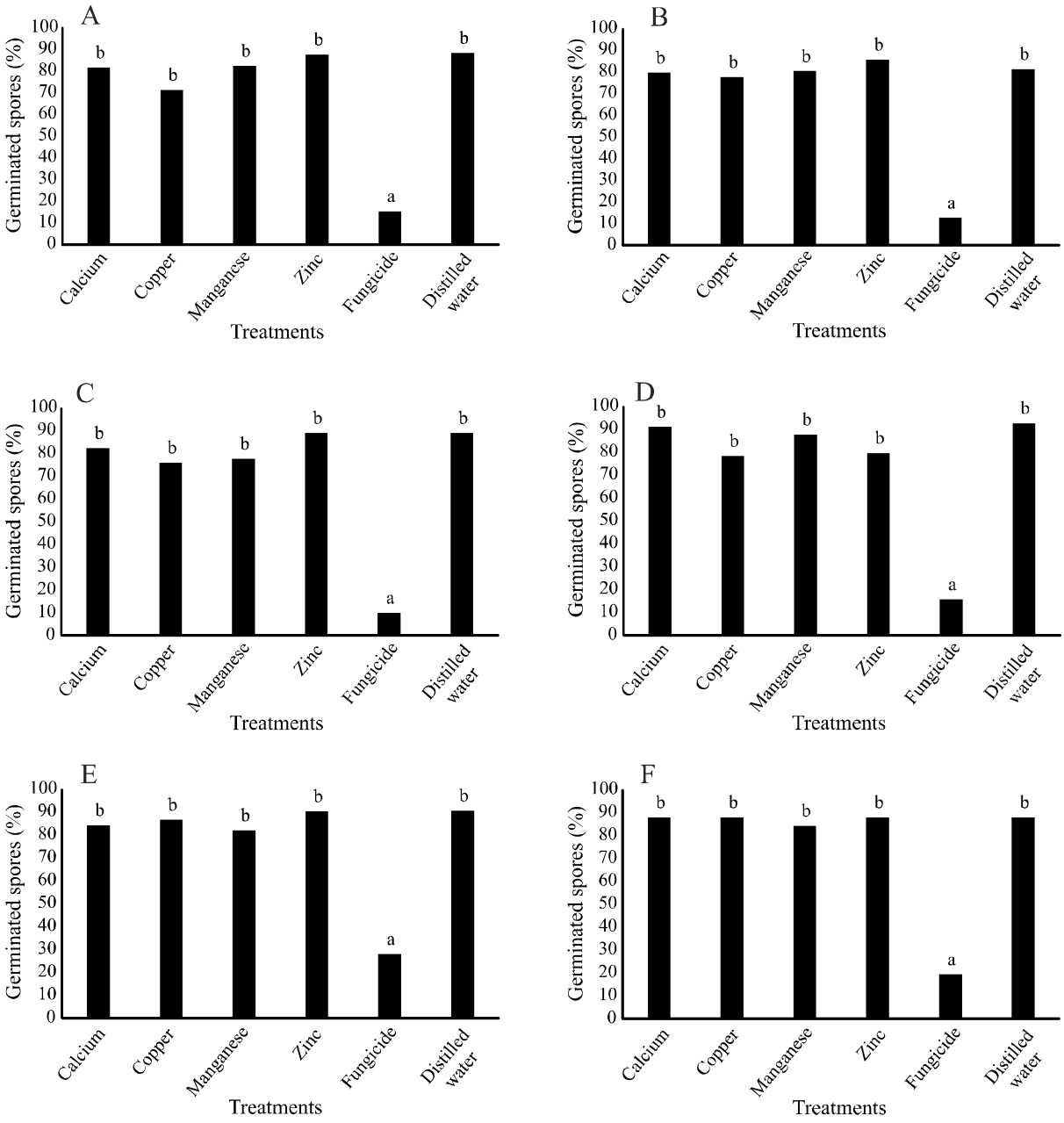
Apparently, treatments with calcium, copper, manganese, and zinc-based products presented toxic action against *Cercospora* sp., preventing the germination of spores when applied at 0, 1, 2 or 3 hours after treatments, and in later times (4 and 5 hours after treatments) the germination of conidia was the same as untreated. This may have occurred because 4 hours after treatment the products, or part of them, had already been absorbed by the plant, which decreased the direct toxic effect. It should be considered, however, that this likely direct fungitoxic effect was higher for uredospores of *P. sorghi* than for the conidia of *Cercospora* sp., as observed in the *in vitro* assay (Figures 1A and 2B), mainly in relation to copper.

For *E. turcicum*, in the same way as observed in the *in vitro* assay, calcium, copper, manganese, and zinc treatments did not present inhibitory effect on the conidia germination of this pathogen for all times evaluated, except for fungicide treatment (Figure 4).

The fungi have simple nutritional needs for survival, being important the use of macronutrients in millimolar and micronutrient in micromolar concentrations. Among the necessary elements, calcium, copper, manganese, and zinc are also required in the growth of fungal cells (WALKER; WHITE, 2017).

These nutrients present different cellular functions, being the calcium possible secondary messenger in the transduction of signals, copper is present in reductor

Figure 4 - Germination of *Exserohilum turcicum* conidia on corn leaf treated with calcium, copper, manganese and zinc -aminoacids based products. Inoculation of the pathogen occurred at the same time as treatment (time zero hours) (A), and 1 (B), 2 (C), 3 (D), 4 (E) or 5 hours (F) after treatments. Columns followed by the same letter do not differ statistically from each other by the Tukey ($p \leq 0.05$). CV (%): 15.19 (A), 7.28 (B), 15.46 (C), 9.94 (D), 7.87 (E) e 7.46 (F)



pigments, and manganese and zinc have functions in the enzymatic activity. Therefore, these compounds are occasionally necessary for specific enzymatic or structural activities of fungi (WALKER; WHITE, 2017). Thus, it may be that some fungal pathogens can perform the absorption of these nutrients using them in their favor and, therefore, there was no decrease in spore germination. According to Griffin (1994), calcium, for example, is an element with function in the activity of some enzymes and in the structure of the fungal membrane, not necessary for all fungi, but required in numerous biochemical processes in major or minor quantity, depending on the genus of the fungus.

Morales, Santos, and Tomazeli (2012) evaluating the effect of the nutrients nitrogen, phosphorus, potassium, sulfur, boron, copper, manganese, molybdenum, zinc and calcium on wheat diseases, observed that the severity of the yellow spot was reduced due to leaf fertilization with them, while for the severity of powdery mildew and rust there was no influence of the treatments.

Thus, considering that pathogens have different strategies to perform pre-penetration and penetration, different nutrients may have distinct needs or toxicity depending on the pathogen, as verified in this study.

CONCLUSION

Calcium, copper, manganese, and zinc aminoacids-based products were able to inhibit *P. sorghi* and *Cercospora* sp. spore germination. When the leaves were treated, for *P. sorghi* percentage of spore germination was influenced by the the time of the treatment. For or *Cercospora* sp. only times zero, 1, 2 and 3 hours after treatment showed influence on the spores germination. Spores of *Exserohilum turcicum* were inhibited only by fungicide treatment *in vitro* and *in vivo*.

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