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Copper(II) compounds as effective agents against *Colletotrichum acutatum* causing anthracnose of strawberry

Original Article

Ivana Stanojević
Faculty of Agriculture, University of Niš, Kosančićeva 4, 37000 Kruševac, Serbia
stanojevic.ivana85@gmail.com

Biljana Glišić
Faculty of Science, Department of Chemistry, University of Kragujevac, R. Domanovića 12, 34000 Kragujevac, Serbia
bglisic@kg.ac.rs

Sanja Živković
Faculty of Agriculture, University of Niš, Kosančićeva 4, 37000 Kruševac, Serbia
gajicsanja43@gmail.com

Sonja Filipović
Faculty of Agriculture, University of Niš, Kosančićeva 4, 37000 Kruševac, Serbia
sonjafilipovic86@yahoo.com

Snežana Andjelković
Institue for forage crops, Kruševac, 37251, Globoder, Serbia
snezana.andjelkovic@ikbks.com

Tanja Vasić
Faculty of Agriculture, University of Niš, Kosančićeva 4, 37000 Kruševac, Serbia
tanjavasic82@gmail.com (corresponding author)

Abstract:

The aim of this study was to evaluate the *in vitro* antifungal effects of copper(II) compounds on the growth of the fungus *Colletotrichum acutatum* isolated from strawberry. The growth inhibition activity of Na_2[Cu(1,3-pddadp)]·$6H_2O$ complex (1,3-pddadp is 1,3-propanediamine-*N,N'*-diacetate-*N,N'*-di-3-propionate anion) against this fungus was compared to those of $CuSO_4·5H_2O$ and Ba_2(1,3-pddadp)·$8H_2O$. Additionally, the effects of these compounds on sporulation level and the number of conidia per 1 mm^2 of colony were investigated. The obtained results showed that the highest percentage of inhibition for mycelium growth was achieved at a concentration of 100 and 200 µg/mL for all applied compounds. The biological activities of the investigated compounds were compared with those for the commercial formulation of fungicide Metod 480 SC (captan).

Key words:

antifugal activity, *Colletotrichum acutatum,* copper(II) compounds, strawberry

Apstract:

Jedinjenja bakra(II) kao efikasni agensi prema gljivi *Colletotrichum acutatum*, uzročniku antraknoze jagoda

Cilj ovog rada je ispitivanje *in vitro* antifungalne aktivnosti jedinjenja bakra(II) prema gljivi *Colletotrichum acutatum* koja je izolovana iz jagode. Ispitivana je aktivnost Na_2[Cu(1,3-pddadp)]·$6H_2O$ kompleksa (1,3-pddadp je 1,3-propandiamin-*N,N'*-diacetat-*N,N'*-di-3-propionat), pri čemu su dobijeni rezultati poređeni sa aktivnošću $CuSO_4·5H_2O$ i Ba_2(1,3-pddadp)·$8H_2O$. Dodatno je analiziran je uticaj različitih koncentracija ovih jedinjenja na intenzitet sporulacije i broj konidija po 1 mm^2 kulture *C. acutatum*. Dobijeni rezultati su pokazali da sva ispitivana jedinjenja imaju najveću aktivnost pri koncentraciji od 100 i 200 µg/mL. Biološka aktivnost ispitivanih jedinjenja je poređena sa odgovarajućom aktivnošću komercijalnog fungicida Metod 480 SC kaptan.

Ključne reči:

antifungalna aktivnost, *Colletotrichum acutatum,* jedinjenja bakra(II), jagoda

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Introduction

Strawberry (*Fragaria ananassa*) is commercially the most important fruit, which is grown worldwide and is predominant species cultivated for production globally (FAO, 2000). One of the species that has been reported to cause wide spread disease in strawberry, namely anthracnose, is the fungus *Colletotrichum acutatum* (Sutton, 1992; Maas and Palm, 1997; Freeman et al., 1998; Wedge et al., 1999). This fungus can attack the plant at all stages of development, including flowers, leaves, stems and roots. However, it causes the greatest damage to fruits, causing significant losses in strawberry production (Mertely et al., 2018).

There are only a few available fungicides which are effective against *C. acutatum*, nevertheless a long and frequent use of these fungicides led to the

resistance development (Maas, 2004). Considering the heavy burden for the strawberry production sector worldwide that anthracnose is causing, development of advanced and efficient therapeutic options became of great importance.

With the increasing emergence of drug-resistant *C. acutatum* species and due to a lack of potent agent against anthracnose, a special attention has been devoted to the evaluation of antimicrobial potential of metal compounds (Schatzschneider, 2019). Among them, copper(II) compounds have received a particular interest, due to their well-known application in agriculture as fungicides, nutritionals and algicides (Richardson, 1997). The importance of copper(II)-based compounds as fungicides in viticulture has been recognized after discovery of the Bordeaux mixture, which consists of copper(II) sulfate, lime and water. Nowadays, the pharmaceutical companies have been produced different copper(II)-based fungicides in soluble forms of sulfates, oxychlorides, acetates, carbonates, oleates, silicates and hydroxides. Copper(II) oxychloride has been widely used due to its good crop tolerance and effectiveness against different phytopathogenic fungi such as *Rhizoctonia solani, R. bataticola, Botrytis cinerea, Fusarium semitectum, F. culmorum, F. moniliforme, F. solani, F. oxysporum, Stemphylium radicinum, Sclerotinia sclerotiorum and Colletotrichum gloeosporioides* (Gharieb et al., 2004). Copper(II)-based fungicides have a broad spectrum of activity against different fungal and bacterial microorganisms as a consequence of their ability to be attached on the plant surface. At the same time, they are economically acceptable and do not lead to resistance development (Müller et al., 2000). With the aim to selectively deliver Cu(II) ion to the diseased tissues, different copper(II) complexes with various ligands have been synthesized and reported to possess significant antibacterial and antifungal activities (Singh et al., 2008; Singh et al., 2009; Patel et al., 2010; Ng et al., 2016).

Beside copper(II) compounds, diaminopolycarboxylate ligands which belong to EDTA family (ethylenediamine-*N,N,N',N'*-tetraacetate anion) and their metal complexes have shown a remarkable antimicrobial activity (Anderegg, 1987; Bulman, 1987; Douglas and Radanović, 1993). For instance, EDTA has shown activity against different Gram-negative and Gram-positive bacteria, yeasts, amoeba and fungi (Brown and Richards, 1965; Kite and Hatton, 2014).

Considering the importance of copper(II) compounds and diaminopolycarboxylate ligands in design of antifungal agents, in the present study, we have synthesized and biologically evaluated $Na_2[Cu(1,3\text{-pddadp})]\cdot 6H_2O$ complex (1,3-pddadp is 1,3-propanediamine-*N,N'*-diacetate-*N,N'*-di-3-propionate anion) against the fungus *C. acutatum*. The obtained biological results for this complex was compared to those for $CuSO_4\cdot 5H_2O$ and $Ba_2(1,3\text{-pddadp})\cdot 8H_2O$.

Material and methods

Materials

Barium salt of diaminopolycarboxylate ligand from EDTA-family, $Ba_2(1,3\text{-pddadp})\cdot 8H_2O$, and copper(II) complex $Na_2[Cu(1,3\text{-pddadp})]\cdot 6H_2O$ were prepared according to a procedure published in the literature (Radanović et al., 1992; Radanović et al., 1997). Other reagents were obtained commercially and used without further purification.

Test organism

In this experiment, the isolate of *C. acutatum* was used. It was isolated from strawberries grown in Serbia. The isolate was determined on the basis of morphological, pathogenic and molecular characteristics and maintained on a potato-dextrose agar (PDA) at 25 °C.

Antifungal activity

Antifungal activity of the three compounds $CuSO_4\cdot 5H_2O$, $Ba_2(1,3\text{-pddadp})\cdot 8H_2O$ and $Na_2[Cu(1,3\text{-pddadp})]\cdot 6H_2O$ was tested on PDA in Petri dishes of 90 mm diameter. Substrates were inoculated by mycelial fragments of *C. acutatum* isolates taken from the edge of 7-days old culture. Tested compounds were dissolved in distilled water at three different concentrations: 100, 200 and 300 µg/mL. The solutions were incorporated into the autoclaved PDA, which was cooled to 55 °C. Cultures prepared in this way were grown for 7 days in a thermostat at 25 °C. The commercial formulation of fungicide Metod 480 SC (captan, Galenika fitofarmacija) was used as a positive control and applied at a concentration recommended for practical application. The negative control variant, fungal isolates grown on PDA, were used under identical conditions without described treatments (Zang et al., 2012). After seven days, the linear increase in the studied cultures was measured. The inhibition of the fungal growth expressed in percentage terms was determined from the growth in the test plate relative to the respective control plate as given below: Inhibition (%) = (C-T) 100 / C where, C = diameter of fungal growth in the control plate and T = diameter of fungal growth in the test plate.

Sporulation level

Ten days after treatment, the effects of $CuSO_4\cdot 5H_2O$, $Ba_2(1,3\text{-pddadp})\cdot 8H_2O$ and $Na_2[Cu(1,3\text{-pddadp})]\cdot 6H_2O$

Table 1. The percentage of growth inhibition of *C. acutatum* by $Na_2[Cu(1,3\text{-pddadp})].6H_2O$, $CuSO_4.5H_2O$ and $Ba2(1,3\text{-pddadp}).8H_2O$ compounds

Compound	100 µg/mL	200 µg/mL	300 µg/mL
$Na_2[Cu(1,3\text{-pddadp})]\cdot 6H_2O$	36.3[b,c]	38.7 [b]	31.6 [c]
$CuSO_4\cdot 5H_2O$	35.3[b,c]	34.7 [c]	39.3 [b]
$Ba_2(1,3\text{-pddadp})\cdot 8H_2O$	39.7 [b]	34.3 [b]	40.0 [b]
Control *C. acutatum*	53.3[a]	53.3 [a]	53.3 [a]
Metod 480 SC	100[d]	100[d]	100[d]

* The data in rows marked by the same letter are not statistically significantly different based on Duncan test (p = 0.05)

on *C. acutatum* sporulation levels were determined. Determination of sporulation levels was performed using a Tahoma haemocytometer. For this purpose, a spore suspension was prepared by adding 5 mL of distilled water to the Petri dish with a culture of isolate *C. acutatum*.

The sporulation level was expressed according to the scale by Quesada and Lopez (1980), where: + = poor sporulation (<5.000 spores/mL), ++ = medium sporulation (5.000-10.000 spores/mL) and +++ = abundant sporulation (>10.000 spores/mL). The experiment was set in three repetitions.

The number of conidia per 1 mm^2 of colony was determined based on the number of conidia/mL and the area of the colony, which was calculated from the proportion of paper weight on which the contours of colonies were drawn and paper weight of 1 dm^2 (Stojanović, 1997).

The results obtained during the research were processed by ANOVA analysis with the statistical program STATISTICA 8.0 (StatSoft Inc., USA). Duncan's test was conducted to analyze the difference between various pre-treatments. A value of P = 0.05 was considered statistically significant.

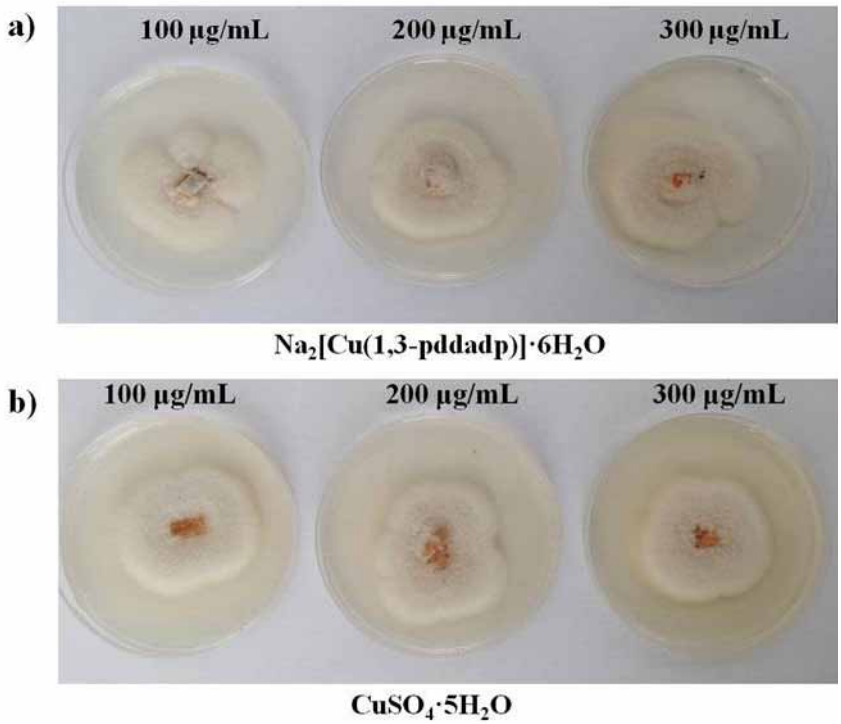

Fig. 1. Influence of two copper(II) compounds, $Na_2[Cu(1,3\text{-pddadp})].6H_2O$ and $CuSO_4.5H_2O$ on the growth inhibition of *C. acutatum* mycelium

Results and discussion

Biological activity

All tested compounds showed a remarkable growth inhibition of *C. acutatum* mycelia. The inhibition of mycelial growth was observed at concentrations of 100 µg/mL and higher, for all tested compounds, $Na_2[Cu(1,3\text{-pddadp})]\cdot 6H_2O$, $CuSO_4\cdot 5H_2O$ and $Ba_2(1,3\text{-pddadp})\cdot 8H_2O$, and was dependent on the applied concentration of the compounds. Moreover, a significant percentage inhibition of *C. acutatum* mycelium compared to the commercial captan formulation (Metod 480 SC, Galenika fitofarmacija) was achieved at all applied concentrations (100, 200 and 300 µg/mL) of the tested compounds (**Tab. 1** and **Figs. 1** and **2**).

The linear growth of colonies of the tested *C. acutatum* isolate was statistically significantly influenced by the concentrations of the applied compounds. During the study, it was found that the

Table 2. Influence of the investigated compounds on the number of conidia per mm² of *C. acutatum*

Compound	100 µg/mL	200 µg/mL	300 µg/mL
$Na_2[Cu(1,3\text{-pddadp})]\cdot 6H_2O$	+	++	+
$CuSO_4\cdot 5H_2O$	+++	++	++
$Ba_2(1,3\text{-pddadp})\cdot 8H_2O$	++	+	+++
Control *C. acutatum*	+++	+++	+++
Metod 480 SC	-	-	-

***** Sporulation: + = poor sporulation, ++ = medium sporulation, +++ = abundant sporulation

highest linear growth of the colony of *C. acutatum* was achieved after treatment with $CuSO_4\cdot 5H_2O$ and $Ba_2(1,3\text{-pddadp})\cdot 8H_2O$ at a concentration of 300 µg/mL relative to the negative control (pure culture of *C. acutatum*) (**Tab. 1**, **Fig. 2b** and **2c**). Contrary to this, the smallest increase in mycelium of the fungus *C. acutatum* was after treatment with $Na_2[Cu(1,3\text{-pddadp})]\cdot 6H_2O$ and $CuSO_4\cdot 5H_2O$ at concentrations of 100 and 200 µg/mL (Fig. 2a and 2b). Considering this, it can be concluded that the minimum inhibitory concentrations (MICs) against the *C. acutatum* are at 100 and 200 µg/mL for the tested compounds (**Tab. 1** and **Fig. 2**).

Oziengbe and Osazee (2012) evaluated the inhibitory effect of copper(II) sulfate on the linear growth of *C. gloeosporioides* mycelium. Their results showed that this copper(II) salt reduced the linear growth of this fungus in all applied concentrations (0.2, 0.4, 0.6 and 0.8 mg/L).

On the other hand, Everett and Timudo-Torrevilla (2007) tested four commercial copper-based preparations (Champ (copper-hydroxide, 37.5% copper), Kocide (copper-hydroxide, 35% copper), Kocide (copper-hydroxide, 46.1% copper) and Cuprofix Disperss (copper-hydroxosulphate, 20% copper)) against the most important pathogens on avocado, *Colletotrichum acutatum, C. gloeosporioides, Botryosphaeria parva, B. dothidea* and *Phomopsis* spp. in New Zealand. The results showed that four copper formulations inhibited the germination of spores of all tested fungi, but at high concentrations (*i.e.* 40.9 µg/mL for copper hydroxide (46.1% copper) against *C. gloeosporioides*). The copper formulation that was effective at the lowest concentrations against the germination of *Botryosphaeria parva* spores was copper hydroxide (37.5% copper), the most effective against both species of *Colletotrichum* was copper-hydroxosulfate (20% copper), while the most

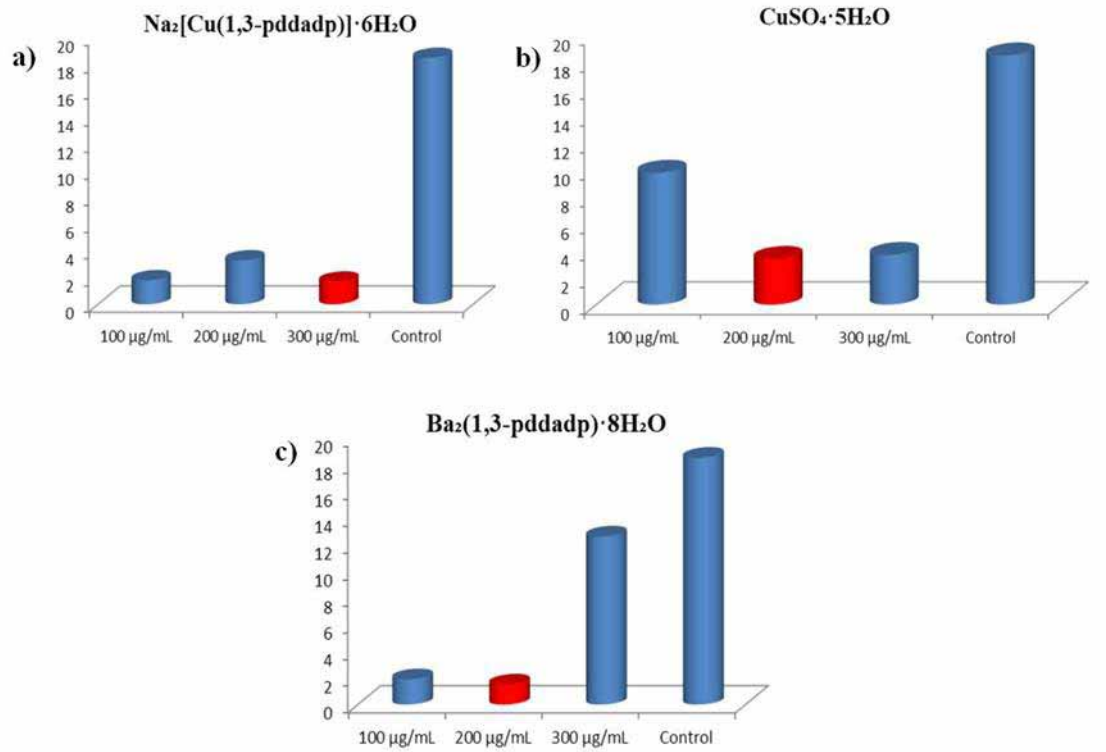

Fig. 2. The effect of $Na_2[Cu(1,3\text{-pddadp})].6H_2O$ complex (a), $CuSO_4.5H_2O$ (b) and $Ba_2(1,3\text{-pddadp}).8H_2O$ (c) on the growth of *C. acutatum* isolate *in vitro* after seven days.

effective against *B. dothidea* and *Phomopsis* sp. was copper-hydroxide (46.1% copper) (Everett and Timudo-Torrevilla, 2007).

Sporulation level

The number of conidia formed per mm^2 of colony was statistically significantly conditioned by the applied concentrations of the tested compounds in *C. acutatum* culture. The lowest number of conidia per mm^2 was found for $Na_2[Cu(1,3\text{-pddadp})]\cdot 6H_2O$ at 100 and 300 µg/mL, for $CuSO_4\cdot 5H_2O$ at 200 and 300 µg/mL, and for $Ba_2(1,3\text{-pddadp})\cdot 8H_2O$ at 100 and 200 µg/mL. The highest number of spores per mm^2 was observed for $CuSO_4\cdot 5H_2O$ and $Ba_2(1,3\text{-pddadp})\cdot 8H_2O$ at concentrations of 100 and 300 µg/mL, respectively (**Tab. 2**).

Table 2. Influence of the investigated compounds on the sporulation level of *C. acutatum*

Compound	100 µg/mL	200 µg/mL	300 µg/mL
$Na_2[Cu(1,3\text{-pddadp})]\cdot 6H_2O$	+	++	+
$CuSO_4\cdot 5H_2O$	+++	++	++
$Ba_2(1,3\text{-pddadp})\cdot 8H_2O$	++	+	+++
Control *C. acutatum*	+++	+++	+++
Metod 480 SC	-	-	-

* Sporulation: + = poor sporulation, ++ = medium sporulation, +++ = abundant sporulation

As can be seen from **Tab. 3**, the ability of the fungus *C. acutatum* to form conidia is affected by different concentrations of the applied compounds, $Na_2[Cu(1,3\text{-pddadp})]\cdot 6H_2O$, $CuSO_4\cdot 5H_2O$ and $Ba_2(1,3\text{-pddadp})\cdot 8H_2O$. In the treatment with captan which was used as a positive control, no sporulation of *C. acutatum* occurred. In the case of $Ba_2(1,3\text{-pddadp})\cdot 8H_2O$ at 200 µg/mL and for $Na_2[Cu(1,3\text{-pddadp})]\cdot 6H_2O$ complex at 100 and 300 µg/mL, a lower and medium level of sporulation was noticed. Contrary to this, the abundant sporulation was observed for the $CuSO_4\cdot 5H_2O$ at 100 µg/mL, as well as for $Ba_2(1,3\text{-pddadp})\cdot 8H_2O$ at 300 µg/mL (**Tab. 3**).

Spores are very important for the spread of the disease on strawberry plantations. Thus, the number of spores and the intensity of sporulation play a significant role in the spread of pathogens and the progress of infection. Spores of the pathogen are passively dispersed by spraying rain or irrigation water which causes infection and pathogen develop on immature fruits and young tissues, and these spores germinate and penetrate the cuticle and epidermis to multiply through the tissues (Babović, 2003). Symptoms include small black spots and/or large black lesions on the tissue surface, which coalesce and penetrate deep into the fruit, resulting in fruit rot.

Borkow and Gabbay (2009) found that different copper formulations have inhibited a spore germination of *Colletotrichum gloeosporioides* at different concentrations (0.2, 0.4, 0.6 and 0.8 mg/L). Similar conclusions were drawn by Oziengbe and Osazee (2012), who tested different concentrations of copper compounds against the isolates of *Colletotrichum gloeosporioides* originated from mango.

Conclusion

In conclusion, the safety of copper for humans and its powerful biocidal properties allow the use of copper and its compounds for different applications. This study showed that copper(II) compounds in the form of complex or simple inorganic salt could inhibit the growth of *Colletotrichum acutatum* mycelium, by affecting the intensity of sporulation and the number of spores per mm^2 *in vitro*, while strongly causing defensive reactions on the strawberry fruit.

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