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ORIGINAL ARTICLE

Inhibition effect of selected inorganic metal ions on the mycelial growth of *Cryphonectria parasitica*

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Abstract

In the current study the antifungal activity of inorganic reagents was tested against *Cryphonectria parasitica in vitro* in a mycelial growth inhibition test. Three reagents, each consisting of chloride silver (AgCl) in combination with (1) aluminum oxide – Al_2O_3, (2) zinc oxide – ZnO, and (3) Al_2O_3 and titanium dioxide – TiO_2, were tested. Significant differences of the tested reagents on the growth of *C. parasitica* were recorded. The study demonstrated that silver in mixture with ZnO had an antifungal effect and significantly reduced the mycelial growth of *C. parasitica in vitro*. The mixture of AgCl with the other two combinations of inorganic metal oxides had no inhibition effect on the growth of the pathogen. It was confirmed that ZnO (applied in a single compound test) is responsible for inhibition of *C. parasitica* mycelium growth. A preliminary in planta assay was performed but statistically significant differences were not recorded in the average increment of canker length.

Keywords: antifungal activity, in planta assay, *in vitro* test, inorganic reagents, silver chloride, zinc oxide

Introduction

The use of inorganic antimicrobial agents has attracted interest for the control of microorganisms and more recently of phytopathogenic fungi (Jo *et al.* 2009; Malachova *et al*. 2011; Silva-Castro *et al.* 2018). The key advantages of inorganic antimicrobial agents are improved safety and stability, which are lacking in organic antimicrobial agents (Jo *et al*. 2009).

One of the most studied inorganic agents is silver since its activity is very broad. It displays multiple modes of inhibitory action to microorganisms, and it may be used for controlling various plant pathogens and managing plant diseases (Jo *et al.* 2009). A broad spectrum of antifungal activity of silver nanoparticles was observed against a wide range of wood-degrading fungi (Narayanan and Park 2014) and various phytopathogenic fungi (Krishnaraj *et al.* 2012; Gopinath and Velusamy 2013; Pinto *et al.* 2013).

Inorganic metal oxides (such as TiO_2, MgO, CaO and ZnO) have been studied as antimicrobial agents because of their safety and stability (Sharma *et al.* 2010).

Zinc is an essential mineral for physiological and metabolic processes of several tissues and organs. It is especially important in the immune system. In addition, Zn is an essential micronutrient for plants and is recommended as fertiliser for crops (Savi *et al.* 2013). On the other hand, it is important to highlight that both too high and too low concentrations of zinc under specific conditions can have toxic effects. For fungi, there are few studies that report the antifungal activity of Zn-compounds, such as against *Botrytis*, *Penicillium* (He *et al.* 2011) and *Fusarium* (Savi *et al.* 2013). The antifungal parameters of ZnO against filamentous fungi, *Aspergillus* and *Rhizopus,* were also identified. ZnO

inhibited fungal growth, but at higher concentrations (Sawai and Yoshikawa 2004).

The antimicrobial behavior of titanium dioxide (TiO_2) has been studied over the years (Gelover *et al.* 2006). It was found to have maximum activity against *Escherichia coli* (Migula) Castellani and Chalmers and minimum activity against the fungus *Candida albicans* (C.P. Robin) Berkhout. The photo catalytic antimicrobial activity was the highest in virus when compared to bacteria or bacterial spores (Kühn *et al.* 2003).

Sadiq *et al.* (2009) investigated the antibacterial effect of aluminium oxide (Al_2O_3) towards *E. coli.* A mild inhibitory effect at high concentrations was detected. In another study the bacterial toxicity of aluminium oxide against *Bacillus subtilis* and microalgae was observed (Mukherjee *et al.* 2011; Sadiq *et al.* 2011).

Chestnut blight disease, a serious tree disease caused by ascomycete fungus *Cryphonectria parasitica* (Murrill) M.E. Barr, is associated with extensive necrosis of the bark. The cambium under the infected bark is killed and the bark appears sunken or swollen. Cracks can also form on the infected surface. Characteristic flat mycelial fans are normally present throughout the bark (EPPO 2005). Chestnut blight is a severe disease responsible for the devastation of chestnut stands in North America since 1904 and Europe since 1938 (Biraghi 1946; Anagnostakis 1987) with significant ecological and economic consequences (Elliott and Swank 2008). In most European countries the chestnut blight epidemic is restricted by hypovirus which infects *C. parasitica* and reduces its virulence. In Slovakia, chestnut blight remains a serious problem since natural hypovirulence of this disease by hypovirulent strains of *C. parasitica* has had little effect so far (Juhásová *et al.* 2005; Adamčíková *et al.* 2019). Similarly, selection of *Castanea* hybrids resistant to chestnut blight (Bolvanský *et al.* 2014; Pažitný *et al.* 2018) has not been. Therefore, there is an interest to find possible modes of protection against the chestnut blight disease to restrict the pathogenic fungus.

Recently, in an attempt to control the growth of *C. parasitica,* chitosan oligomers, propolis and silver nanoparticles individually and their mixtures in solution were tested in *in vitro* experiments (Silva-Castro *et al.* 2018). From the tested reagents only chitosan oligomers and propolis had inhibition effects on *C. parasitica*. The effectiveness of chitosan oligomers both with and without propolis was around 93%. An individual propolis solution also had a high inhibition effect (64%, Silva-Castro *et al.* 2018).

Generally, in forest pathology, there are only a few studies regarding the use of inorganic metal oxides in protecting trees against fungal disease. In this study the antifungal activity of inorganic reagents was assessed against the fungal pathogen *C. parasitica* through laboratory and preliminary in planta assays.

Materials and Methods

Fungal isolates, reagents

Two virulent isolates of *C. parasitica* were used for the test. One isolate was isolated from *Castanea sativa* Mill., of local origin (Krupina, Slovakia). The second, M1285, originated from a European database of vegetative-compatible (vc) types (Gnosca, Switzerland) (Cortesi *et al.* 1998). Fungal strains were maintained in the culture collection of the Institute of Forest Ecology SAS Zvolen, Department of Plant Pathology and Mycology. For the experiment the isolates were grown on 3% malt extract agar (MEA) in Petri dishes for 7 days at 23°C in the dark.

Three reagents, each consisting of chloride silver (AgCl) in combination with different inorganic ingredients containing modified (1) aluminum oxide – Al_2O_3, (2) zinc oxide – ZnO, and (3) Al_2O_3 and titanium dioxide – TiO_2, were tested. All reagents used for *in vitro* tests were supplied by Ansil, s.r.o. (Nová Dubnica, Slovakia). The following commercial products were used: Ansilver®-A, Ansilver®-Z, Ansilver®-A/T.

Later, the single inorganic compound was tested for the reagents that significantly reduced the growth of the pathogen to determine the antifungal effect of individual compounds or the interaction of two compounds.

Laboratory assay

The antifungal assay was performed *in vitro* on 9 cm Petri dishes as a mycelium growth inhibition test with two different methods of reagent application. In the first method the reagents were added directly into the cultivation media before sterilization. The second method involved using a micropipette to evenly apply the suspension of reagents on the surface of solid cultivation media after its sterilization.

Initially the same concentration ($0.5 \text{ g} \cdot 100 \text{ ml}^{-1}$) of all tested reagents was used to select the reagent which would have an impact on fungal growth. Later, different, lower concentrations ($0.25 \text{ g} \cdot 100 \text{ ml}^{-1}$, $0.125 \text{ g} \cdot 100 \text{ ml}^{-1}$ and $0.05 \text{ g} \cdot 100 \text{ ml}^{-1}$) of a selected reagent (which showed an inhibition effect) were tested. Each treatment combination was replicated five times.

Lastly, a single inorganic compound test was carried out to determine the involvement of individual compounds of a reagent on fungal growth. In this test four different concentrations were tested ($0.2 \text{ g} \cdot 100 \text{ ml}^{-1}$, $0.1 \text{ g} \cdot 100 \text{ ml}^{-1}$, $0.04 \text{ g} \cdot 100 \text{ ml}^{-1}$, $0.01 \text{ g} \cdot 100 \text{ ml}^{-1}$), with nine replications per concentration.

For tests, 3% MEA was used. Two central lines at right angles were drawn on the bottom of each Petri dish. One cube of agar ($5 \times 5 \text{ mm}^2$) cut from the margin of actively growing fungal mycelium was placed in the middle of each Petri dish. They were then incubated on

a laboratory bench at room temperature in daylight. As a negative control, isolates of *C. parasitica* were grown on 3% MEA without any application of the studied reagents.

The mycelial diameters (d_1, d_2) were measured along predetermined lines three times. The mycelial area was calculated with the following formula:

$$\text{Mycelial area} = d_1 \times d_2 \times \pi/4$$

The effect of the reagents used on mycelial growth of *C. parasitica* was tested with Repeated measures ANOVA using software STATISTICA 10.

In planta assay

The antifungal activity of compounds that had a significant effect on the mycelial growth of *C. parasitica* was tested *in vivo* on 5-year-old chestnut seedlings growing in pots. Fourteen seedlings were artificially inoculated with isolate M1285, one inoculation hole/seedling stem. The length of cankers was measured 35 days after inoculation. The outer bark on the canker was carefully removed to identify the border of the canker and to expose the mycelium of *C. parasitica*. The tested compound was mixed with LacBalsam® Wound Sealing (Frunol delicia® GmbH, Germany; 0.1 g of compound/50 ml LacBalsam) and applied directly on the mycelium in the canker. Treatment was carried out on seven seedlings. The other seven plants were used as negative control, covered only with LacBalsam without any supplement. The length of cankers was remeasured 45 days after the tested compound application. The effect of treatment was evaluated by comparing the canker increment. Data were tested with one-way ANOVA using software STATISTICA 10.

Results

There were no differences in mycelial growth between the two isolates of *C. parasitica* ($F = 0.0125$; $p = 0.912$). The average mycelial area for isolate M1285 was 33.07 mm^2, and for the local isolate it was 32.45 mm^2. Also, the mycelial growth rate over time for the two tested isolates was similar ($F = 0.18$; $p = 0.83$). No statistical differences were recorded between the isolates, therefore in further analyses the results for individual isolates were pooled.

The mode of application had a significant effect on the mycelial growth of *C. parasitica* ($F = 7.68$; $p = 0.024$). The application of tested reagents directly into the media reduced the fungal growth more effectively than the application of reagent suspension on the surface of solid cultivation media (Fig. 1).

There were significant differences between tested reagents on the growth of *C. parasitica* ($F = 90.1$; $p < 0.01$). The most inhibitive of the three reagents on the mycelial growth was the reagent containing ZnO. The next two reagents (containing Al_2O_3 (1) and $Al_2O_3 + TiO_2$ (3)) had an insignificant effect compared to the negative control; the mycelial growth was similar to the negative control (Fig. 2).

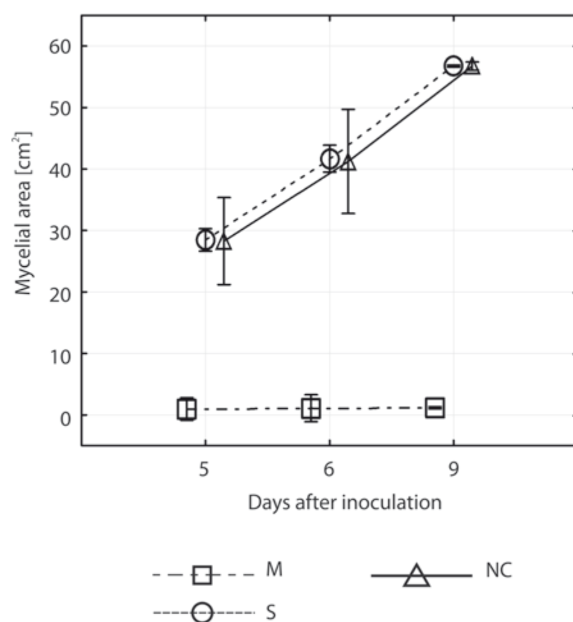

Fig. 1. Significant effects of the mode of application on the mycelial growth of *Cryphonectria parasitica* (M = application directly to medium, S = suspension applied on the surface of solid medium, NC = negative control). Vertical bars denote 0.95 confidence intervals

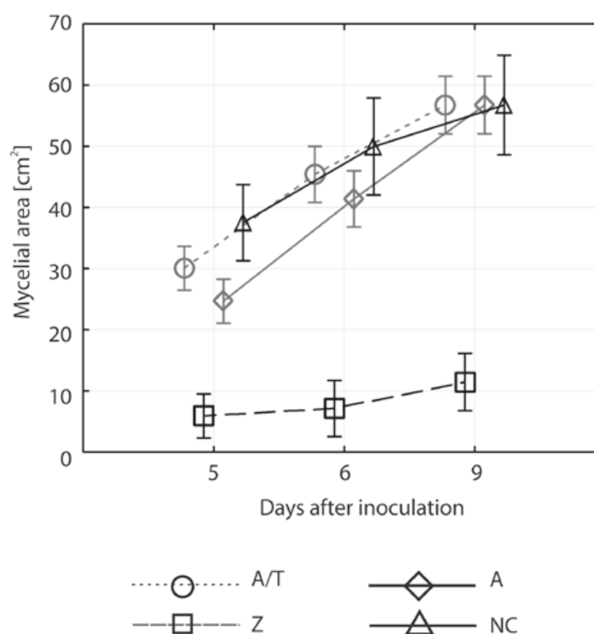

Fig. 2. Inhibition effects of three tested reagents with different inorganic compounds on the mycelial growth of *Cryphonectria parasitica* in *in vitro* tests (tested reagents: A/T = AgCl + Al_2O_3 + TiO_2, Z = AgCl + ZnO, A = AgCl + Al_2O_3, NC = negative control). Vertical bars denote 0.95 confidence intervals

For the subsequent test only reagent (2), AgCl + + ZnO, which had an inhibition effect, was assessed at different, lower concentrations. All lower concentrations of reagent applied directly into the media had a significantly inhibitive effect on the mycelial growth of *C. parasitica* ($F = 10981.6$; $p < 0.01$; Fig. 3). At concentrations 0.25 g and 0.125 g · 100 ml^{-1} the inhibition effect was similar ($p = 0.279$). At the lowest concentration (0.05 g · 100 ml^{-1}) the inhibition effect was somewhat lower than the two higher concentrations ($p < 0.01$), but they were significantly different in comparison to the negative control ($p < 0.01$; Figs. 3 and 4A). When applied on the media surface the inhibition effect was not evident, and it did not differ significantly from the negative control at all tested concentrations.

One single inorganic compound test, ZnO, was tested. The tested concentrations were equivalent to its concentration in previous tests in reagents as binary reagent with AgCl and one markedly lower concentration was chosen. A significant inhibition effect was seen for three higher concentrations (0.2 g · 100 ml^{-1}, 0.1 g · 100 ml^{-1} and 0.04 g · 100 ml^{-1}, $F = 265.2$; $p < 0.01$) with a greater inhibition effect with increasing concentrations of ZnO. At the lowest concentration

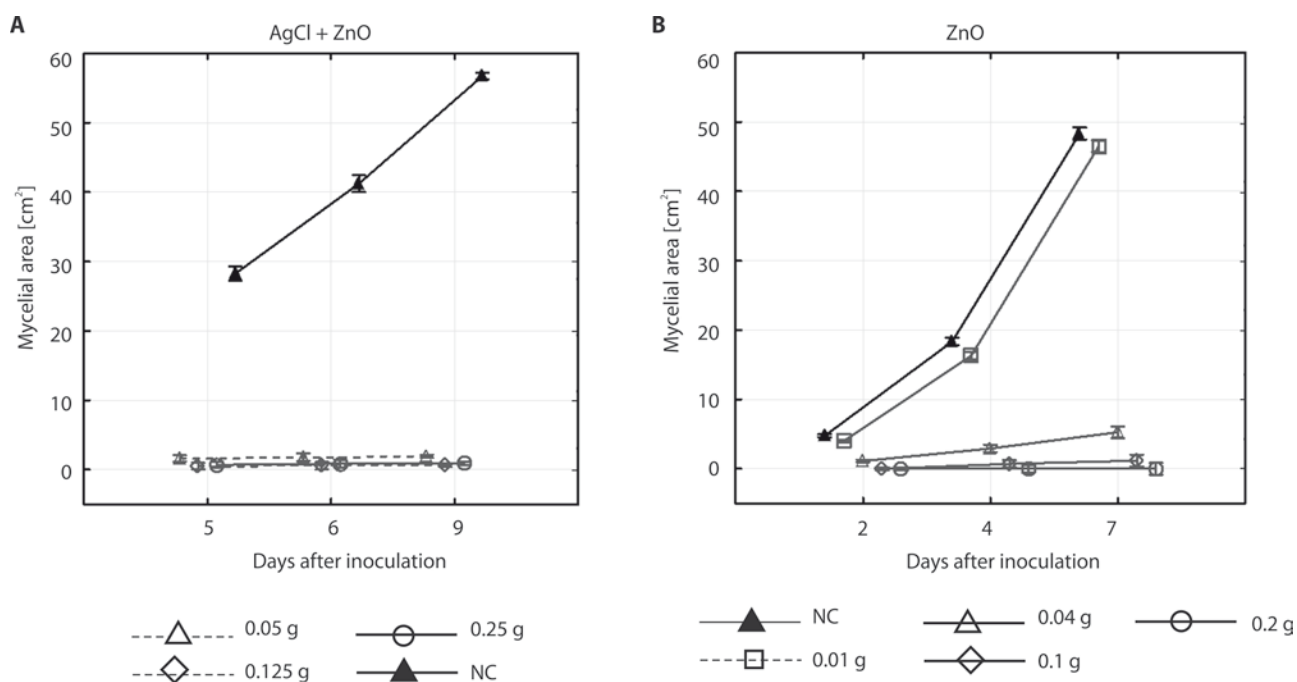

Fig. 3. Antifungal effects of binary compound AgCl + ZnO (left) and single compound ZnO (right) tested at different concentrations against *Cryphonectria parasitica* added directly to medium. All tested concentrations had significant effects on mycelial growth except one, the lowest concentration (0.01g · 100 ml^{-1}) for ZnO. NC = negative control. Vertical bars denote 0.95 confidence intervals

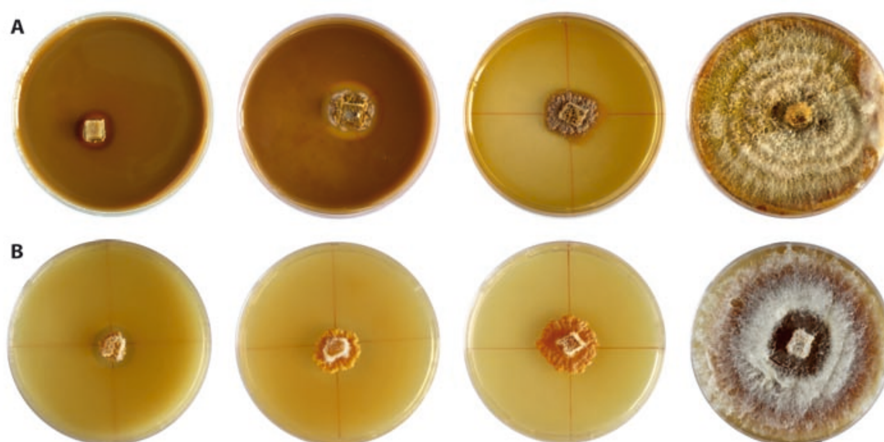

Fig. 4. Antifungal effects of (A) binary compound AgCl + ZnO and (B) single compound ZnO tested at different concentrations against *Cryphonectria parasitica* added directly into the cultivation medium, *in vitro* growth inhibition test on Petri dishes left to right: (A) different concentrations of reagent 0.25 g, 0.125 g and 0.05 g · 100 ml^{-1}, negative control, (B) 0.2 g, 0.1 g, 0.04 g and 0.01 g · 100 ml^{-1}

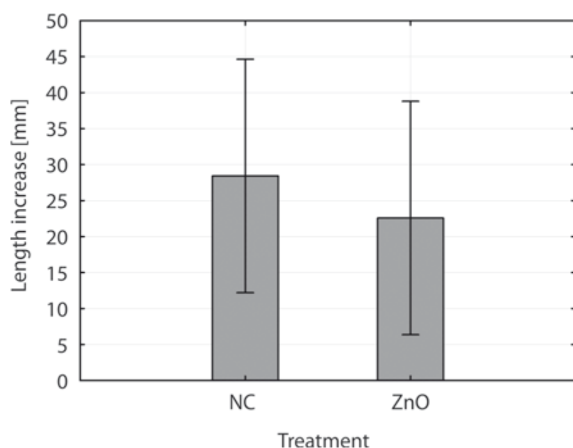

Fig. 5. In planta assay of antifungal activity of ZnO. Average increment in canker length between chestnut blight cankers treated with tree balsam mixed with ZnO and negative control (NC). Vertical bars denote 0.95 confidence intervals

(0.01 g · 100 ml^{-1}) an insignificant effect of ZnO on mycelial growth was recorded. No difference was noticed in mycelial growth in comparison to the negative control ($p = 0.108$; Figs. 3 and 4B).

In planta assay no statistically significant differences were recorded in the average increment in canker length between chestnut blight cankers treated with ZnO and the negative control ($F = 0.007$; $p = 0.94$) in spite of a visually noticeable higher increment in canker length for the negative control (average increment = = 28.43 mm) compared to the cankers treated with ZnO (average increment = 22.57, Fig. 5).

Discussion

The management of fungal diseases on food and ornamental plants is economically important. The present study demonstrated that from tested inorganic reagents, AgCl in mixture with ZnO had antifungal effects and significantly reduced the mycelial growth of *C. parasitica in vitro*. The mixture of AgCl with the other two combinations of inorganic metal oxides had no inhibition effect on pathogen growth.

Silver has been investigated and employed more extensively than any other inorganic antibacterial agent. One of the potential applications in which silver can be utilized is in management of plant diseases. The efficiency of silver antifungal activity is affected by the silver compound, so the form of silver is significant. $AgNO_3$ showed a higher antifungal activity (Jo *et al.* 2009). Although AgCl has also been known to have antimicrobial activity (Simonetti *et al.* 1992), it is much less effective than silver ions for inhibiting colony formation and suppressing disease development (Jo *et al.* 2009). All three reagents used in our study contained silver in AgCl, which should explain the negative results.

On the other hand, Silva-Castro *et al.* (2018) did not find the same significant inhibition effect of silver nanoparticles on the growth of *C. parasitica*, even though the silver was prepared in an aqueous solution of $AgNO_3$. These results indicate that silver has no or little effect on mycelial growth of *C. parasitica*. The anti-fungal activity of the treatments depends on the particular type of microorganism (Silva-Castro *et al.* 2018). While silver ions did not inhibit the growth of *C. parasitica* and some other tested fungal pathogens such as *Gremeniella abietina*, *Heterobasidion annosum* (Silva-Castro *et al.* 2018), they are highly effective in reducing the mycelial growth of other species such as *Fusarium circinatum* and *Diplodia pinea* (Silva-Castro *et al.* 2018), *Bipolaris sorokiniana* and *Magnaporthe grisea* (Jo *et al.* 2009). Antifungal properties of different forms of silver often coexisting in solution have not been investigated fully (Jo *et al.* 2009). Recent studies (Silva-Castro *et al.* 2018) investigated the effect of silver in some binary and ternary mixtures which had a counter-productive effect on the antifungal activity against some fungal pathogens of trees.

In the present study binary and ternary mixtures of silver were investigated, which were selected as the existing commercial product. Separately, as a single, individual inorganic compound, ZnO was tested for its inhibition of mycelial growth with the aim to see if ZnO is the primary agent responsible for the inhibition effect. The present study demonstrated that a single, individual compound, ZnO, at three out of four tested concentrations had an inhibition effect on the growth of *C. parasitica* mycelium *in vitro*.

Malachová *et al.* (2011) investigated the antifungal activity of montmorillonite (MMT), Ag-MMT, Cu--MMT and Zn-MMT. On the tested wood-decay fungi silver ions had the lowest inhibition. This means that the antifungal effect of copper and zinc ions was significantly higher than the inhibition by silver ions which is in contrast with the strong antibacterial activity of silver ions (Malachová *et al.* 2011). This finding is consistent with the results of our study which confirmed the antifungal effect of Zn on the mycelial growth of fungal pathogens.

Whereas low concentrations of some metal ions including copper and zinc ions are necessary for fungal growth, silver does not belong to essential elements. This fact can explain the differences between the ability of Ag^{+}, Cu^{2+} and Zn^{2+} to penetrate into fungal cells (Malachová *et al.* 2011). Cu^{2+} and Zn^{2+} are transported by the uptake system for essential metal ions to the cell where they can accumulate and exert toxic effects at high concentrations (Baldrian 2010). This could explain the no inhibition effect of the lowest concentration of ZnO on mycelial growth. Generally, the mechanism of the biocidal action of metal ions involves disrupting the membrane and leads to the death

of the microorganism (Padmavathy and Vijayaraghan 2008), causing oxidative damage in proteins, inducing morphological changes and influencing reproduction (Baldrian 2010). In fungi they are far from being understood in detail (Malachová *et al.* 2011).

Primary requirements for the potential use of inorganic agents in plant disease control include more information about antifungal activity of various compounds to plant pathogens and the development of better application strategies to increase the efficacy of disease suppression (Jo *et al.* 2009). In conclusion from the *in vitro* growth inhibition experiments it could be inferred that not only the binary compounds, AgCl + ZnO, but also the single, individual compound ZnO have significant antifungal effects on chestnut blight fungus.

In planta experiments the antifungal activity of reagents is rarely tested. The majority of tests are performed in vitro in Petri dishes. In the present study the inhibition effect of ZnO in planta assay was not confirmed. This assay needs to be better developed. The reasons for the failure of the planta assay can be several. The way of ZnO application on the cankers (in the present study the compound was applied mixed in tree balsam) could have a significant impact. The presently used form of ZnO application could have an impact on the efficiency of its absorption and penetration to the cells. The application of ZnO in solid form (mixture with tree balsam) could be less effective than in liquid form, similar to soil application of Zn fertilizers used in field agronomic management (Du *et al.* 2014). Zinc solubility of the suspended ZnO in either fine powder or granular form is very low (<3 mg Zn · l^{-1}) and to compensate this shortcoming, very high concentrations of ZnO are often required (Du *et al.* 2014). Also, the application timing, preventive or after fungal colonisation, should be investigated. For example, in planta efficacy, in the case of silver ions, it is much greater with preventative application, which may promote the direct contact of silver with spores and germ tubes, and inhibit their viability (Jo *et al.* 2009). Another important aspect is the possible uptake of ZnO by plant tissues since it is an important micronutrient element for plants. This kind of possible interaction should also be studied to set up an effective mode of application.

Additionally, further research is needed to test and develop the application strategy of studied compounds in planta experiments, especially to focus on mode, technique and timing of application. It is also important to find the effective concentration of compounds for field application.

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