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Tolerance and Bioaccumulation of Copper by the Entomopathogen *Beauveria bassiana* (Bals.-Criv.) Vuill. Exposed to Various Copper-Based Fungicides

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Abstract This work evaluates for the first time the relationships between copper-tolerance, -solubilization and -bioaccumulation in the entomopathogen *Beauveria bassiana* exposed to Bordeaux mixture, copper oxychloride or copper hydroxide. Bordeaux mixture was highly detrimental to fungus, by inhibiting the growth totally at the recommended dose (RD) and 2×RD. Copper hydroxide and copper oxychloride were found to be less toxic, reducing fungus growth, sporulation and conidial germination in an average of 29 %, 30 % and 58 %, respectively. These two copper forms were the easiest to solubilize, to precipitate and the most accumulated by *B. bassiana*, suggesting the involvement of all these processes on fungus copper-tolerance.

Keywords *Beauveria bassiana* · Copper-based fungicides · Tolerance

Copper-based fungicides are frequently used by farmers, several times per year, to control plant fungal diseases (Soares et al. 2006). Among these preparations, copper sulfate, copper oxychloride and copper hydroxide are the most frequently used in the world. Their active compound is copper ion (Cu^{++}) which acts by direct contact, inducing denaturation of enzymes and proteins of the cell membrane of the spores and mycelium leading to a general disruption of metabolism and cell integrity. It also inhibits spore germination (Gisi and Sierotzki 2008).

The successful use of copper-based preparations depends on their compatibility with other crop protection strategies. Their compatibility with entomopathogenic fungi is of particular concern to farmers. Copper-based preparations have a broad spectrum of activity and therefore can adversely affect the efficacy of entomopathogenic fungi. In Portugal, the presence of entomopathogenic fungi has been recently observed, with *Beauveria bassiana* (Bals.-Criv.) Vuill. being the most abundant species (Oliveira et al. in press). This fungus is one of the most promising biocontrol agents against a great number of agricultural insect pests (Akello et al. 2009), also having the ability to inhibit the growth of several phytopathogenic fungi (Ownley et al. 2008).

Knowledge of the effect of commercial copper-based fungicides that are currently used by the farmers on *B. bassiana* is scarce. A number of studies have focused on the effects of non-copper fungicides on *B. bassiana* survival, growth or efficacy (Olmert and Kenneth 1974; Loria et al. 1983; Majchrowicz and Poprawski 1993; Todorova et al. 1998; Jaros-Su et al. 1999; Shapiro-Ilan et al. 2002; Kouassi et al. 2003; Shah et al. 2009). Only a few experiments aimed at the effects of copper-based fungicides on *B. bassiana* have been carried out (Olmert and Kenneth 1974; Bååth 1991; Majchrowicz and Poprawski 1993; Jaros-Su et al. 1999; Tamai et al. 2002; Kouassi et al. 2003). The results obtained showed that the susceptibility of *B. bassiana* to the fungicides varied depending on the chemical used (Olmert and Kenneth 1974; Majchrowicz and Poprawski 1993; Jaros-Su et al. 1999; Shah et al. 2009), the timing of application (Jaros-Su et al. 1999;

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Kouassi et al. 2003), the dosage of active ingredient (Majchrowicz and Poprawski 1993; Shah et al. 2009) and the experimental conditions under which evaluation of the fungi was tested, e.g. in vitro or field conditions (Loria et al. 1983; Jaros-Su et al. 1999). In general, copper-based fungicides have shown to be less toxic to the fungus than non-copper fungicides (Olmert and Kenneth 1974; Jaros-Su et al. 1999). However, some copper-based fungicides have been observed to be highly toxic to *B. bassiana*, such as copper oxychloride (Majchrowicz and Poprawski 1993; Tamai et al. 2002) and copper oxide (Kouassi et al. 2003).

The main aim of this study was to evaluate, under in vitro conditions, the tolerance of an autochthonous *B. bassiana* isolate to three copper-based fungicides currently used for managing plant fungal diseases, Bordeaux mixture, Cupravit and Kocide, with respective active ingredients of copper sulphate, copper oxychloride and copper hydroxide. The effects of the dosage of active ingredient on *B. bassiana* fungicide-tolerance were evaluated and the possible mechanisms involved in fungicide-tolerance will be also evaluated for the first time.

Materials and Methods

An isolate of *Beauveria bassiana* (A39GF09) was obtained from the fungal culture collection of the School of Agriculture, Polytechnic Institute of Bragança, Portugal. This isolate was originally obtained in 2009, from field-collected mycosed pupae of *Prays oleae* from olive groves located in the Trás-os-Montes region (northeast of Portugal). Fungus isolation was performed on potato-dextrose agar (PDA) and after obtaining a pure culture, the isolate was identified by amplification and sequence of the internal transcribed spacer region (ITS), using the universal primers *ITS1* and *ITS4* (White et al. 1990). The fungus was maintained in an aqueous glycerol solution (30 %, v/v) at −20°C (Oliveira et al. 2010).

Working cultures of *B. bassiana* were prepared from frozen stock by transferring conidia, with a bacteriological loop, from the aqueous glycerol solution previously thawed onto Petri dishes with PDA medium. The dishes were incubated in the dark at 25°C for at least 7 days until colonies with multiple conidia were produced. *B. bassiana* conidia were then collected by flooding fungal cultures with 2 mL of 0.02 % (v/v) Tween 80 sterile solution. The number of conidia per mL in the obtained conidial suspension was counted in a Thoma counting chamber and further used as inoculum in the present study.

The influence of copper based-fungicides on *B. bassiana* growth was evaluated on PDA medium amended with three different concentrations, at recommended dose (RD), half (1/2 RD) and double (2 RD) of the recommended dose, of each the fungicides Bordeaux mixture (BM), Cupravit (CO) and KOCIDE® 35 DF (CH). These fungicides belong to the same chemical class (inorganic compounds) and their active ingredient is copper from copper sulphate (20 w/w) for the BM, copper oxychloride (50 w/w) for the CO and copper hydroxide (35 w/w) for the CH. The fungicides were mixed into PDA medium post-autoclaving, whilst the medium was still liquid (i.e., 50 ± 5°C). The quantities of fungicides added into the culture medium were: (1) at recommended dose, 15.0 g/L for BM, 5.0 g/L for CO and 3.5 g/L for CH; (2) at half of the recommended dose, 7.5 g/L for BM, 2.5 g/L for CO and 1.8 g/L for CH; (3) and at double of the recommended dose, 30.0 g/L for BM, 10.0 g/L for CO and 7.0 g/L for CH. The control treatment was performed in the same medium without fungicides. After mixing well, 10 mL of each medium was poured onto 9.0-cm-diameter Petri dishes and an autoclaved cellophane membrane was placed aseptically on the surface of the agar. Each Petri dish was centrally inoculated with 5 μL of *B. bassiana* conidial suspension containing 3×10^6 conidia/mL, sealed with Parafilm and incubated in the dark at 25°C. Ten replicate dishes were prepared for each fungicide concentration. The radial growth of the developing colony was measured every 4 days, using two cardinal diameters previously drawn on the bottom of the dish, for 12 days. Growth inhibition (in percentage) was calculated relatively to control and radial growth rate (mm/day) was also determined.

Twelve days after incubation, the viability and the production of conidia by *B. bassiana* in each treatment (fungicides and concentrations) were evaluated. For the conidia production, a conidia suspension was retrieved from fungus cultures, obtained in the growth assessment, and placed into 500 μL of an aqueous solution of Tween 80 (0.02 %, v/v). The number of conidia was counted in a Thoma counting chamber. Results were expressed in conidia per mL. The viability was determined by quantifying the percentage of germinated conidia. Thereafter, 100 μL of the spore suspension used to quantify conidia, at the concentration 6×10^6 (conidia/mL), was spread in 9-cm Petri dishes containing agar medium (15 g/L agar–agar). Ten replicates of each fungicide per concentration were performed. After incubation, at 25 ± 1°C in the dark for 18 h, the percentage of germination was evaluated microscopically by counting the number of germinated spores, from a total of 300 spores per Petri dish. Only the conidia with germ tubes longer than their width were considered to have germinated.

Macroscopic characteristics of *B. bassiana* colonies were registered during the 12 days of fungal culture. These included mycelium texture, the colour of the colony and its outline, medium coloration and exudates production.

Copper content on *B. bassiana* mycelium and culture media was determined after 12 days of fungal growth in

each treatment (fungicides and concentrations). The *B. bassiana* mycelium was then removed from the surface of the cellophane; and both the mycelium and the culture media were dried separately in a ventilated oven at 30°C for 2 days. Three replicates were prepared for each fungicide and concentration. The dried mycelia and culture media were reduced to powder with a mortar and pestle, and approximately 0.05–0.50 g (mean weight) were weighed and transferred to a closed Teflon container for acid digestion (HNO_3 + HCl) during 17 h in a stove thermostatically controlled at 110°C to completely dissolve the sample. The digested solution was transferred to a decontaminated tube and diluted to a convenient volume with doubly deionized water. Copper quantification was carried out in a Perkin-Elmer model AAnalyst 600 Spectrometer with Zeeman background correction, equipped with an AS-800 autosampler and a HL-2040 Printer. Analyses were performed using Perkin-Elmer THGA tubes and WinLab32 control software. The furnace program was used with an ashing temperature of 1,200°C and an atomization temperature of 2,000°C. The copper quantification method was fully validated as previously indicated for other biological matrices (Soares et al. 2006). Linearity was observed in the range of 0.19–20.0 µg/L. The detection limit and the quantification limit were calculated as the concentration corresponding to three/ten times the standard deviation (SD) of the background noise and the values found were, respectively 0.19 and 0.63 µg/L. Then, on the basis of 0.05 g for mycelium or 0.50 g for culture media in a 10 mL final volume, the quantification limits were, respectively 126 and 12.6 µg/Kg. This was calculated as the concentration corresponding to ten times the standard deviation of the background noise. All of the solutions were prepared with doubly deionized water and the chemicals used (HCl, HNO_3) were of pro analysis grade (Merck). Standard copper solutions were prepared daily from 1,000 mg/L solution (Spectrosol, BDH) in 0.2 % HNO_3 Suprapure grade (Merck).

Data from radial growth of colonies, mycelium dry weight (g), spore germination (%), number of spores (conidia/mL) and copper contents (%) are presented as the mean of three or ten independent experiments displaying the respective SE bars or SD values. Differences among means were determined by analysis of variance (ANOVA), using SPSS v.18 software and averages were compared using Tukey's ($p < 0.05$). *Spearman* correlation coefficients were determined using the same software package.

Results and Discussion

The results obtained showed that the fungicides and the concentrations tested had a significant influence on the growth of *B. bassiana* (Fig. 1). Among all of the fungicides used in the experiment, the Bordeaux mixture showed the greatest inhibitory effect on the growth of *B. bassiana*, followed by copper oxychloride and copper hydroxide. The Bordeaux mixture inhibited fungus growth at concentrations equal to or the double the RD. Although copper oxychloride and copper hydroxide allowed the fungus to grow in all of the concentrations tested, they significantly reduced *B. bassiana* growth. Copper oxychloride significantly reduced *B. bassiana* growth in all of the concentrations tested, whereas copper hydroxide only significantly inhibited fungus growth at the highest concentration (i.e., double the RD). Growth of *B. bassiana* was highly correlated with fungicide concentration, displaying a significantly negative effect. This result was confirmed by the *Spearman* correlation coefficients (Table 1). This agrees with the results of Majchrowicz and Poprawski (1993) and Shah et al. (2009), who verified decrease in entomopathogenic fungal growth as the concentration of active ingredient of fungicide increased. This dose-dependent fungicide effect was more evident for Bordeaux mixture, with the highest *Spearman* correlation value, than for copper oxychloride and copper hydroxide (Table 1). Thus,

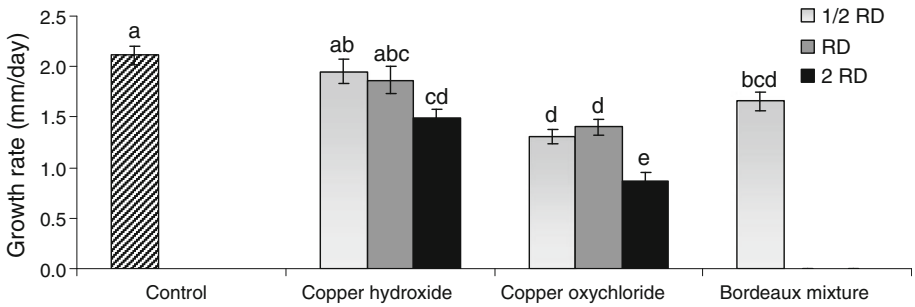

Fig. 1 Growth rates of *Beauveria bassiana* grown on PDA medium unamended (control) or amended with three different concentrations, at recommended dose (RD), half (1/2 RD) and double (2 RD) of the recommended dose, of each the fungicides copper hydroxide, copper oxychloride and Bordeaux mixture, at day 12. Each value is expressed as mean ± standard error (n = 10). Bars with different letters indicate values with significant differences at $p < 0.05$

Table 1 *Spearman* correlation coefficients between concentrations of each the fungicides (copper hydroxide, copper oxychloride and Bordeaux mixture) and number of spores, spore germination, mycelium dry weight, growth rate of *B. bassiana* and contents of copper in mycelium

	Copper hydroxide	Copper oxychloride	Bordeaux mixture
No spores (conidia/mL)	−0.46***	−0.41**	−0.82***
Spore germination (%)	−0.62***	−0.73***	−0.93***
Mycelium dry weight (g)	ns	ns	−0.84***
Growth rate (mm/day)	−0.47***	−0.70***	−0.89***
Copper in mycelium (%)	0.33*	0.82***	0.83***

Statistical significance: *ns* not significative; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

our results show that *B. bassiana* presents several susceptibilities to the tested fungicides, depending on the active ingredient used. Copper oxychloride and copper hydroxide showed a fungistatic effect, whereas Bordeaux mixture a fungicidal effect. Previously, it was similarly found that copper hydroxide had little inhibitory effect on *B. bassiana* (Olmert and Kenneth 1974; Jaros-Su et al. 1999) compared to copper oxychloride (Majchrowicz and Poprawski 1993; Tamai et al. 2002). Information of Bordeaux mixture effects on entomopathogenic fungi is not available in the literature compared to the other two copper-based fungicides tested in this study. The majority of published works focused the effect of Bordeaux mixture applications on fungal and bacterial disease management (Agrios 2005).

The effect of copper-based fungicides on *B. bassiana* growth was also different over the duration of the incubation (Fig. 2). During the first 8 days of incubation, all of the fungicides and concentrations tested significantly reduced *B. bassiana* growth. Afterwards, growth was significantly inhibited by Bordeaux mixture and copper oxychloride at all the concentrations tested. For copper hydroxide, the concentrations corresponding to ½ RD and RD did not cause any significant effect on fungus growth when compared to control, although significant growth inhibition was observed for the highest concentration. These results suggested that toxicity of copper hydroxide is reduced with increased incubation time beyond 8 days. By contrast, Bordeaux mixture and copper oxychloride seemed to maintain their toxicity against *B. bassiana* over the entire incubation. Earlier studies have also shown a reduction in the toxicity of fungicides as days progress following their application (Rachappa et al. 2007).

In this study, it was verified that the copper-based fungicides tested, as well as their concentrations, affected negatively both sporulation and conidial germination (Table 2). Copper hydroxide significantly reduced spore production by 32 % at the RD and by 50 % at 2RD, as compared to control; whereas copper oxychloride only exhibited a negative effect on *B. bassiana* sporulation at 2RD (55 % spore reduction). Conidial germination was more negatively affected by the fungicides (*Spearman* correlation coefficients ranging from −0.62 to −0.93) than

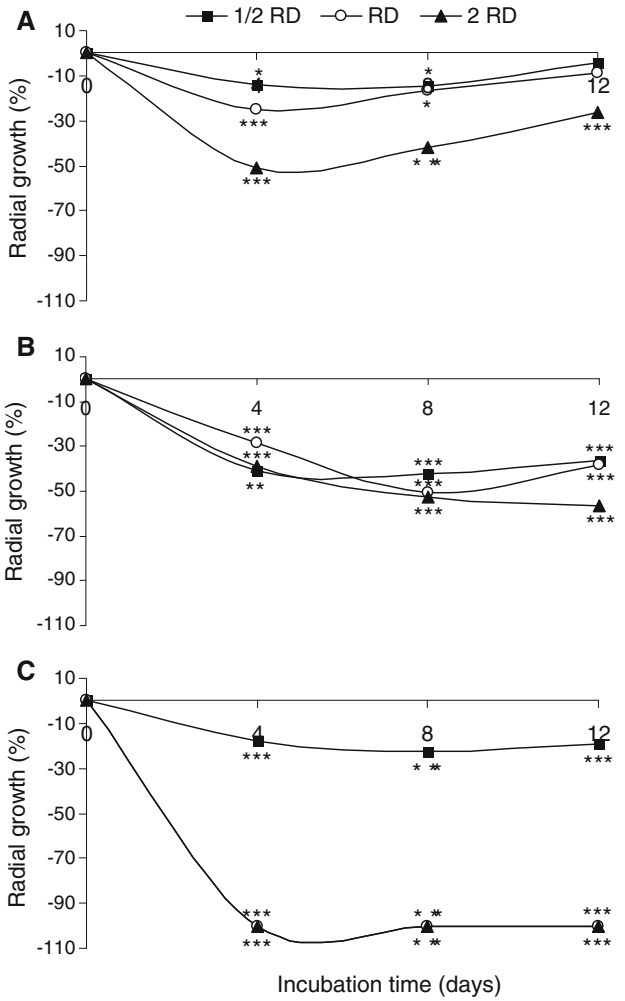

Fig. 2 Differences of radial growth (%) of *Beauveria bassiana* cultured on PDA medium unamended and amended with three different concentrations, at recommended dose (RD), half (1/2 RD) and double (2 RD) of the recommended dose, of each the fungicides copper hydroxide (**A**), copper oxychloride (**B**) and Bordeaux mixture (**C**), for 12 days. Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

sporulation (*Spearman* correlation coefficients ranging from −0.41 to −0.82) (Table 1). Germination percentages decreased significantly with all fungicides at the concentrations tested, ranging from 57 % to 63 % for copper

hydroxide and from 56 % to 62 % for copper oxychloride. Of the fungicides tested, Bordeaux mixture was the most fungistatic to *B. bassiana*, with a germination inhibition of 75 % at ½ RD. At concentrations equal to or double the recommended dose, this fungicide completely inhibited conidial germination, explaining the absence of fungal growth in these treatments. Similarly, the decrease in conidia germination by copper oxychloride and copper hydroxide may explain the decline in vegetative growth observed in *B. bassiana* cultured in medium amended with these fungicides. The obtained results suggest that each copper-based fungicides has a different mode of action; and that their application could restrict the potential of *B. bassiana*, since inhibition of conidia germination could prevent infection of the target host. Information on the effect of fungicides, and especially of copper-based fungicides, on sporulation and conidial germination of *B. bassiana* are scarce in the literature. In the few studies that have been conducted, copper hydroxide and copper oxychloride had no significant effects on *B. bassiana* sporulation (Jaros-Su et al. 1999) or conidial germination (Durán et al. 2004), respectively, in either laboratory or field conditions. In the present study we observed contradictory responses which could be due to inherent variability of chemicals to individual fungal species.

Table 2 Conidia production and conidial germination (mean ± SD, n = 10) of *Beauveria bassiana* cultured on PDA medium unamended (control) and amended with three different concentrations, at recommended dose (RD), half (1/2 RD) and double (2 RD) of the recommended dose, of each the fungicides copper hydroxide, copper oxychloride and Bordeaux mixture, at day 12

Treatment	No conidia ($\times 10^6$conidia/mL)	Conidial germination (%)
Control	26.8 ± 17.1^{ab}	67.4 ± 13.0^{a}
Copper hydroxide		
½ RD	57.6 ± 29.4^{a}	28.8 ± 2.3^{b}
RD	18.1 ± 4.5^{c}	26.5 ± 4.6^{b}
2 RD	13.5 ± 9.6^{c}	24.9 ± 9.4^{b}
Copper oxychloride		
½ RD	61.5 ± 26.4^{a}	29.9 ± 1.7^{b}
RD	45.6 ± 15.9^{ab}	25.5 ± 2.0^{b}
2 RD	12.1 ± 4.4^{c}	27.5 ± 2.4^{b}
Bordeaux mixture		
½ RD	30.7 ± 18.9^{bc}	16.7 ± 6.6^{c}
RD	n.d.	n.d.
2 RD	n.d.	n.d.

In each row different letters mean significant differences ($p < 0.05$)
n.d not determined

This work has clearly demonstrated that *B. bassiana* was able to solubilize all the insoluble inorganic copper-based fungicides, which was noticed by the production of a clear zone (halos) underneath and around colonies growing on medium amended with those fungicides (Fig. 3). Copper oxychloride and copper hydroxide showed higher halo radius than Bordeaux mixture (data not shown), which

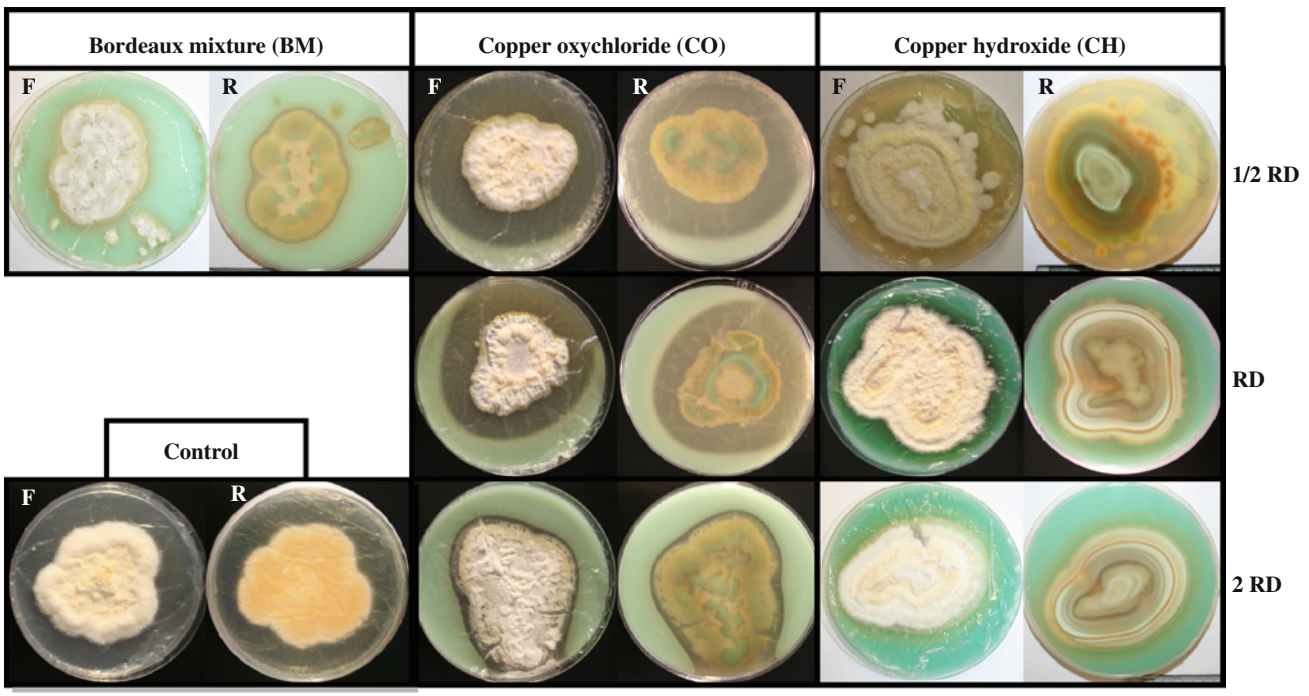

Fig. 3 Macroscopical aspects of *Beauveria bassiana* grown on PDA medium unamended (control) or amended with three different concentrations, at recommended dose (RD), half (1/2 RD) and double (2 RD) of the recommended dose, of each the fungicides copper hydroxide, copper oxychloride and Bordeaux mixture, at day 12. *F* front view, *R* reverse view

suggested that the two first fungicides were much more easily solubilized by *B. bassiana* than Bordeaux mixture. It was also noticeable that, the solubilization of fungicides decreased as their concentrations increased in the culture medium. Only two examples of solubilization of the fungicide copper oxychloride have been described for *Aspergillus niger* and *Penicillium chrysogenum* (Gharied 2002; Gharied et al. 2004), and none for Bordeaux mixture or copper hydroxide. The mechanism of copper oxychloride solubilization by *A. niger* and *P. chrysogenum* were reported to be mediated by organic acids (possibly citric acid), the production of which seems to be induced followed adsorption of the fungicide onto the fungal cells (Gharied 2002; Gharied et al. 2004). Similarly, the excretions of citric acid and mainly of oxalic acid, by the entomopathogenic fungus *Beauveria caledonica*, have been previously observed to play an important role in mineral solubilization (Fomina et al. 2005b). Taking into account all these studies, the solubilization of copper-based fungicides by *B. bassiana* could be related to the production of these compounds. In fact, the presence of copper has been described to stimulate organic acid production by fungi (Clausen and Green 2003).

During growth of *B. bassiana* in the presence of copper-based fungicides, we observed the formation of concentric rings underneath the colonies, which was not verified in control colonies (Fig. 3). On the medium containing copper hydroxide, the concentric rings were white–gray and brownish green, and formed in higher number than in the colonies grown in the medium with copper oxychloride or Bordeaux mixture. In the last two fungicides, only one greenish concentric ring was visible. A similar pattern of concentric rings was observed in *B. caledonica* grown in the presence of copper phosphate, which was related to the precipitation of copper oxalate hydrate crystals (Fomina et al. 2005b).

The type of fungicides and their concentration significantly affects the accumulation of copper by *B. bassiana* (Fig. 4). The highest concentrations of copper were found in mycelia grown in the medium with copper oxychloride (up to an average of 14,269-fold when compared to control, considering the three levels of fungicide tested), followed by Bordeaux mixture (up to 2,950-fold) and copper hydroxide (up to 1,466-fold). The *Spearman* correlation coefficients values determined corroborated these results, being the highest values found for copper oxychloride (Table 1). The capacity of *B. bassiana* mycelia to accumulate copper was further confirmed by calculation of the bioconcentration factor (BCF) values (Fig. 4), calculated as the quotient between the concentration of copper in the mycelium and the concentration in the respective culture medium (on the basis of dry weight). Very high BCFs for copper oxychloride was found, attaining mean values of

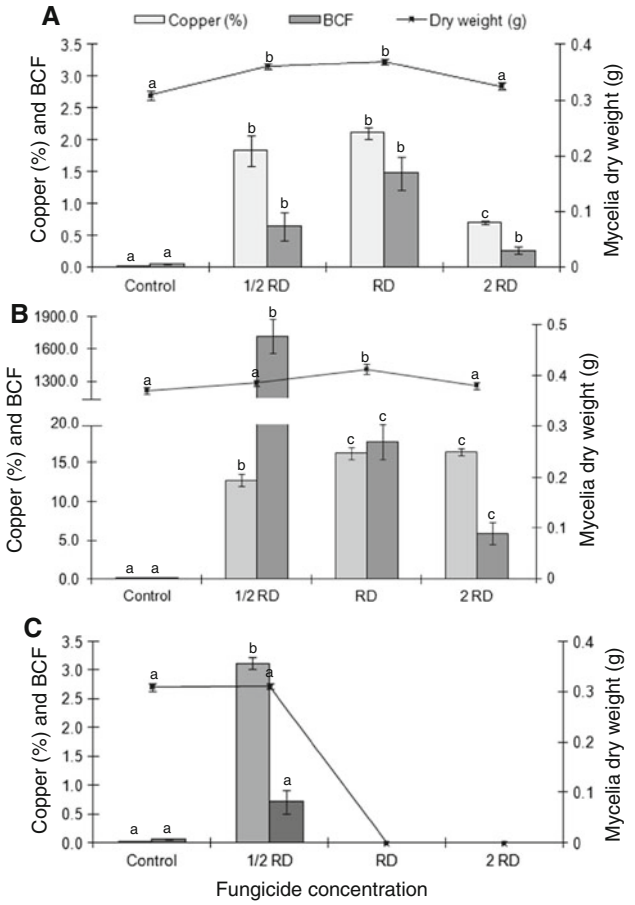

Fig. 4 Contents of copper, bioconcentration factors and mycelia dry weight of *Beauveria bassiana* cultured on PDA medium unamended (control) and amended with three different concentrations, at recommended dose (RD), half (1/2 RD) and double (2 RD) of the recommended dose, of each the fungicides copper hydroxide (**A**), copper oxychloride (**B**) and Bordeaux mixture (**C**), at day 12. Each value is expressed as mean ± SE (n = 3–10). *Bars* with *different letters* indicate values with *significant differences* at $p < 0.05$, within each parameter

580.8 (considering the three concentrations of fungicide tested). There was also a significant increase in mycelium copper content with increased fungicide concentration up to the recommended dose (RD). For the highest doses of fungicides (2RD), copper content in *B. bassiana* decreased. It is known that fungi are able to accumulate significant amounts of metals (Gadd 1993; Figueiredo et al. 2007; Baptista et al. 2009), including entomopathogenic fungi (Popowska-Nowak et al. 2004; Fomina et al. 2005b).

Although *B. bassiana* was able to solubilize copper-based fungicides, and therefore increase copper bioavailability and toxicity, this strain has shown high tolerance to those fungicides, especially to copper hydroxide and copper oxychloride. This clearly indicates possession of copper tolerance mechanisms by this fungus strain. Copper tolerance of fungi has been ascribed to diverse mechanisms

involving biosorption of the metal to cell surface, active uptake of copper, extracellular chelation or precipitation by secreted metabolites, and intracellular complexation (Cervantes and Gutierrez-Crona 1994). The copper tolerance displayed by our fungus strain could be related with the complexation of the mobilized toxic copper by chelating agents excreted by *B. bassiana*, such as protons and/or various metabolites (including organic acids), followed by their precipitation. For copper hydroxide, the precipitation of copper compounds appears to occur mainly in the agar (noticed by the formation of concentric rings), whereas for copper oxychloride this precipitation seems to occur on the fungal mycelium, due to the high levels of copper found on mycelia grown in the presence of this fungicide. Therefore, solubilization of the fungicide copper oxychloride and copper hydroxide, as well as copper-precipitation and/or copper-accumulation in *B. bassiana* mycelium, might be an active process necessary for copper tolerance. In fact, both copper oxychloride and copper hydroxide exhibited the least toxicity, were the easiest to solubilize and the most precipitated and/or accumulated by *B. bassiana*. However, this hypothesis needs further confirmation. Previous studies have also revealed a possible relationship between metal solubilization activity and tolerance (Gharied 2002; Fomina et al. 2005a, b). In a study of metal toxicity towards ericoid- and ectomycorrhizal fungi, it was shown that metal tolerant fungal strains solubilized toxic metal minerals (e.g. Cd, Cu, Pb, Zn) better than non-tolerant isolates (Fomina et al. 2005a). Similarly, Fomina et al. (2005b) reported that metal dissolution by *B. caledonica*, was strongly associated with metal accumulation. In general, with the more metal-tolerant fungal strains, higher biomass yields were registered and more metal dissolution occurred, as compared with the less tolerant strains (Fomina et al. 2004). Moreover, tolerance of *A. niger* to copper oxychloride has also been reported to be correlated with fungicide solubilization and accumulation of copper in mycelia (Gharied 2002).

The findings of this study may help farmers to select appropriate fungicides for use with *B. bassiana*. These results are also of potential environmental and biotechnological importance as *B. bassiana* could have applications in some bioremediation and bioleaching processes due to its high toxic metal tolerance.

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