



## Production of lytic enzymes and siderophores, and inhibition of germination of basidiospores of *Moniliophthora (ex Crinipellis) perniciosa* by phylloplane actinomycetes <sup>☆</sup>

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### ABSTRACT

*Streptomyces albovinaceus*, *Streptomyces caviscabies*, *Streptomyces griseus*, *Streptomyces setonii*, and *Streptomyces virginiae* selected as antagonists of *Moniliophthora (ex Crinipellis) perniciosa*, the causal agent of cacao Witches' broom, were examined *in vitro* to detect production of chitinases,  $\beta$ -1,3-glucanases, and cellulases. All the species produced chitinases, but not  $\beta$ -1,3-glucanases or cellulases, when grown on a liquid mineral medium containing glucose, colloidal chitin, or cell walls of *M. perniciosa* as a carbon source. There were no quantitative differences among species in the production of chitinase, however, the germination inhibition of basidiospores of *M. perniciosa* was higher when they were cultivated using glucose as a carbon source, followed by colloidal chitin and cell walls. All the species also produced hydroxymate type siderophores in similar quantities, and the quantity of siderophores did not correlate with the inhibition of basidiospore germination. The germination inhibition was more pronounced when *S. albovinaceus*, *S. griseus*, and *S. virginiae* were cultivated on iron-deficient medium, suggesting involvement of siderophores in the antagonism by these species of actinomycetes.

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### 1. Introduction

Biological control of plant diseases can be attained through reduction of inoculum quantity or disease causing capacity of a pathogen with use of one or more organisms except man (Cook and Baker, 1983). A microorganism can exert antagonism towards a plant pathogen directly by producing substances acting directly on one or more stages of the life cycle of the pathogen (Cook and Baker, 1983) or indirectly by activating mechanisms of host resistance towards the pathogen (Van Loon et al., 1998).

Iron ( $\text{Fe}^{3+}$ ) is biologically important being a constituent of cytochrome and others heme or non-heme proteins and also a co-factor in various enzymes. When aerobic or facultative anaerobic microorganisms grow in an iron-deficient environment, they synthesize  $\text{Fe}^{3+}$  ion specific chelating agents called siderophores (Goto, 1990). Siderophores are low molecular weight (500–1000 Da) compounds produced by fungi and bacteria, which bind with  $\text{Fe}^{3+}$  ions to be transported into the cell (Neilands, 1989). Based on their structure,

the majority of the known siderophores have been grouped either as catecholates, produced only by bacteria, or as hydroxymates which are produced by fungi and bacteria. The production of siderophores by the biocontrol agents in quantities sufficient may to limited  $\text{Fe}^{3+}$  availability to the pathogen (Glick and Bashan, 1997) and is possible lead to induction of host resistance against the pathogen (Meziane et al., 2005).

The cell wall is responsible for maintenance of physical integrity of cells especially, in hypotonic environments, which predominate in the biosphere (Caldwell, 2000). In higher fungi, the cell wall is composed of layers of chitin and  $\beta$ -1,3-glucan, while cell walls of those of the organisms in the Chromista kingdom, such as oomycetes, are composed of cellulose and  $\beta$ -1,3-glucan (Alexopoulos et al., 1996). The lysis of the cell walls leads to leakage of cell contents and collapse.

Several microorganisms produce lytic enzymes, which can degrade cell wall of other organisms (Gomes et al., 2001). The production of lytic enzymes by fungi or bacteria has been shown to be one of the mechanisms of biocontrol of plant diseases (Kaur et al., 2005). Chitinolytic microorganisms are considered to be more effective antagonists of fungal pathogens because of the direct action of chitinase alone (Yu et al., 2008) or in combination with other antifungal compounds produced by the antagonist (De Boer et al., 1998).

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Witches' broom of cacao, caused by *Moniliophthora perniciosa* (Stahel) Aime and Phillips-Mora (= *Crinipellis perniciosa* (Stahel) Singer), causes heavy losses in cacao (*Theobroma cacao* L.) plantation in Brazil. The fungus invades all the meristematic tissues of the plant (Scarpari et al., 2005). In the Southern region of Bahia State, Brazil, losses of up to 70% caused by the disease were recorded in the last 14 years following its outbreak in 1989 (Andebrhan et al., 1999).

The use of resistant genotypes has shown good results, but 90% of the plantations in the region still consist of susceptible genotypes. Promising results with the biocontrol agent *Trichoderma stromaticum* Samuels and Pardo-Schultheiss, have been reported (Souza et al., 2006). This antagonist parasitizes the pathogen's hyphae before it produces secondary inoculum (basidiocarps) in a plantation (Bastos, 1996). The basidiospores of *M. perniciosa* can be over long distances by winds (Evans and Solorzano, 1981) and infect even *T. stromaticum* treated plants. Thus, it is important to develop a strategy that can protect the susceptible tissues from invasion by the pathogen.

Actinomycetes constitute a morphologically diverse group, distinguished from other Gram-positive bacteria by their filamentous growth and GC-rich DNA (Lacey, 1997). Although actinomycetes are present in both terrestrial and aquatic environments (Goodfellow and Williams, 1983). Several studies have reported the isolation and application of actinomycetes on the plant root system (Silva et al., 2004), endophytes (Conn et al., 2008), and the influence of secondary metabolites on the control of plant diseases (Prabavathy et al., 2006). Several actinomycetes produce antimicrobial compounds able to inhibit the growth of other bacteria, fungi, and protozoa (Ensign, 1992). For this reason, they are being studied with the objective of obtaining new antibiotics (Madigan et al., 2000). These organisms have a great capacity to survive in adverse environments (McBride and Ensign, 1987). It was shown that the ability to accumulate high endogenous concentrations of trehalose is correlated to the capacity of these organisms to resist dry conditions due to the preservation of membrane integrity by this substance (Crowe et al., 1984).

Macagnan et al. (2006) have obtained 203 isolates of endosporegenic bacteria and 94 isolates of actinomycetes from the surface of cacao fruits and showed that 5 actinomycetes strongly inhibited *M. perniciosa* basidiospore germination on fruit surface during the susceptible stage. Preliminary tests on the mode of action of these actinomycetes showed that their culture filtrates were as inhibitory to basidiospore germination as the live cells (Macagnan et al., 2006).

The objectives of this study were to determine the production of siderophores and several lytic enzyme, chitinases,  $\beta$ -1,3-glucanases, and cellulases, by these actinomycetes, and to investigate the involvement of siderophores and chitinases in inhibiting germination of basidiospores of *M. perniciosa*.

## 2. Materials and methods

### 2.1. Origin, storage, and culture of the antagonists

The 5 actinomycetes, originally codified as Ac4, Ac19, Ac26, and Ac68 e Ac79 used in this study were selected and previously described by Macagnan et al. (2006). Based on the rDNA 16S sequence, these isolates were identified as *Streptomyces albobinaceus* (Kudrina, 1957; Pridham et al., 1958), *S. cavaicabies* (Goyer et al., 1996), *S. griseus* (Krainsky, 1914; Waksman and Henrici, 1948), *S. setonii* (Millard and Burr, 1926; Waksman, 1953), and *S. virginiae* (Grundy et al., 1952), respectively. All isolates were stored in 15% glycerin in an ultra-freezer ( $-80^{\circ}\text{C}$ ) in Biological Control of Plant Disease—Viçosa Federal University, Minas Gerais-Brazil (Ghera, 1994). Spores of each isolate were produced on soil-extract agar

(Pramer and Schmidt, 1964), which were then used to seed different substrates for cultivation as described below.

The basidiospores of *M. perniciosa* were obtained by the method of Frias et al. (1995). Dead cacao branches infected by the pathogen were incubated in a moisture saturated atmosphere at  $25^{\circ}\text{C}$  with 12 h photoperiod. Lighting was supplied by a fluorescent lamp. The basidiocarps were harvested and treated with streptomycin sulfate solution ( $150\text{ g mL}^{-1}$ ) by a minute, rinsed in sterile distilled water, and then their upper surface was attached, with use of silicon vaseline, to the inner face of a culture plate cover. The cover was then placed, face down over a beaker containing 15% glycerin in 0.195% MES buffer (Sigma 3671), pH 7.0 and incubated at  $25^{\circ}\text{C}$  with constant stirring. The basidiospores ejected from the basidiocarps were collected in the solution for a period of 18 h. After determining basidiospore concentration in the collecting solution, and their viability rate, the solutions were stored in an ultra-freezer ( $-80^{\circ}\text{C}$ ) until used. The concentrations of basidiospores in the suspensions were adjusted to  $10^6\text{ mL}^{-1}$ , with at least 80% germination for all tests described below.

### 2.2. Detection of lytic enzymes

Each of the 5 isolates was spot-seeded on a mineral agar medium containing 0.5% laminarin (Sigma L9634), carboxymethyl cellulose (Sigma C4146) or 0.08% colloidal chitin to detect  $\beta$ -1,3-glucanases, cellulases or chitinases (Renwick et al., 1991), respectively. The colloidal chitin was prepared according to Reid and Ogrydziak (1981). Non-colloidal chitin (Sigma C9213) (25 g) was milled with help of Universal Mill M20 (Ika, Werke GmbH, Staufen, Germany) and sieved. The fraction retained on the sieve between 0.15 and 0.075 mm screens was digested for 24 h in 85%  $\text{H}_3\text{PO}_4$  at  $4^{\circ}\text{C}$ . The suspension was diluted to 2 L with distilled water and blended in an electric blender. The mixture was centrifuged and the pellet was resuspended in distilled water and blended again. The process was repeated until the water pH reached 5.5, at which time 1 mol NaOH was added to adjust the pH to 7.0. The mixture was centrifuged again and the pellet of colloidal chitin was lyophilized.

For qualitative detection of chitinases, glucanase, and cellulases, the isolates were incubated on the respective culture medium for 10 or 4 days, respectively, at  $28^{\circ}\text{C}$ . There were four replications for each test of each antagonist. The experiment was done twice.

### 2.3. Quantification of chitinase production on different substrates

Each isolate was cultivated on modified Simmons (1926) basal medium, in which the citrate was substituted with 1% glucose, colloidal chitin or cell walls of *M. perniciosa* as carbon source. The cell walls were prepared according to Wisniewski et al. (1991). The fungus was grown for 7 days on potato dextrose broth (Tuite, 1969) and the collected mycelium was washed several times in distilled water and then disintegrated for 2 min with a tissue disintegrator (Ultra-turrax Ika, Werke GmbH, Staufen, Germany) and then frozen overnight. The mycelial suspension was homogenized again and centrifuged for 15 min at 5000g. The supernatant was discarded, the pellet was resuspended in distilled water and homogenized and centrifuged as described above. The process was repeated six times, and then the cell wall preparations were lyophilized.

After 48 h incubation at  $28^{\circ}\text{C}$  the microbial cells were separated from the medium by centrifugation. The supernatant of each treatment was used to quantify chitinase activity by colorimetric determination of Remazol Brilliant Violet 5R liberated from Chitin Azure (Sigma C3020) (Hackman and Goldberg, 1964). The reaction mixture consisted of 900  $\mu\text{L}$  Chitin Azure (1% (w/v) in 50 mmol phosphate buffer, pH 6.0) and 100 L of the culture supernatant. In the control, the supernatant was substituted by the centrifuged culture medium. After 48 h reaction at  $25^{\circ}\text{C}$  under constant stir-

ring, the reaction mixture was centrifuged and the absorbance of supernatant was determined at 575 nm (Hitachi U-3000). After calculating the difference between absorbance of the sample and the control, the data were expressed as units of chitinase. One unit of chitinase corresponded to an increase of 0.001 in the absorption value/day of reaction/g protein. The protein concentration in the supernatant was determined according to Bradford (1976). There were three replications for each treatment. Data were subjected to analysis of variance in a factorial design of  $3 \times 5$  (3 carbon sources and 5 isolates). This experiment was done twice.

#### 2.4. Chitinase and inhibition of germination of basidiospores

The culture supernatant obtained from the growth of the microorganism on purified cell wall, chitin, and glucose was tested for its effect on the germination of basidiospores of *M. pernicioso*. An aliquot (1 ml) of the supernatant was serially diluted using PBS buffer (phosphate buffer 100 mmol pH 7.0 prepared on 0.85% NaCl) (1:1–v:v), six times, and 20  $\mu$ L of each dilution and 10  $\mu$ L of the basidiospore suspension were mixed and placed on water agar in a culture plate. In controls, the supernatant was substituted by the centrifuged culture medium. After 3-h incubation at 25 °C, the proportion of germinated basidiospore was determined microscopically. From each treatment were evaluated 100 basidiospores and was considered to have germinated if the germ tube length was equal to or greater than width of the basidiospore. There were three replications for each treatment. Data were subjected to analysis of variance in  $3 \times 6$  factorial design (3 carbon sources and 5 isolates and a control). This experiment was conducted twice.

#### 2.5. Detection of siderophores

For this experiment, all glassware were cleaned by immersion in dichromate-acid solution for 48 h and then rinsed several times with distilled water before use.

The 5 isolates were shake-cultured, in triplicate, for 48 h in King's B (KB) broth (King et al., 1954). For controls, the medium was amended with 2  $\mu$ mol L<sup>-1</sup> of Fe<sup>3+</sup> provided by the filter sterilized FeSO<sub>4</sub>·7H<sub>2</sub>O solution. The cells were removed from the medium by centrifugation (3600g for 10 min) and 1 mL of the supernatant was mixed with one mL of the Chromo-azuroil S solution (Aldrich 199532) prepared according to Schwyn and Neilands (1987). The change of the mixture color from bluish to reddish brown within 15 min indicated the presence of siderophores. This test was repeated three times.

#### 2.6. Detection and quantification of different forms of siderophores

The hydroxamate type siderophores were detected according to Calvente et al. (2001). The spores of each antagonist were added to semi-solid molten KB medium and distributed into four cavities of dilution plates. The medium, amended to contain 2  $\mu$ mol L<sup>-1</sup> of Fe<sup>3+</sup>, served as controls. These plates were placed in 150-mm Petri plate and incubated for 48 h at 28 °C. This was followed by forming 2 mm diameter wells in the centre of each cavity, to which was added a drop of the reagent described by Atkin et al. (1970). Formation of dark halo, in 10–15 min, around the cavity indicated presence of hydroxamate siderophores. This assay was repeated for four times and the evaluation was done qualitatively.

Catecholate siderophores were detected according to Arnow (1937). The antagonists were grown in shake cultures for 48 h at 28 °C, in triplicate, in KB broth amended or not with 2  $\mu$ mol L<sup>-1</sup> of Fe<sup>3+</sup>. The cells were removed as described previously and 1 ml of the supernatant was added to an equal volume of Arnow (1937) reagent. Development of a reddish color indicated presence of catecholate type siderophores.

The amount of siderophores produced by each of the isolates was quantified according to Schwyn and Neilands (1987), a universal assay, and expressed as equivalents of EDTA (disodium salt). A standard curve of EDTA was constructed by serially diluting a 10 mmol aqueous solution of EDTA. Schwyn and Neilands (1987) reagent was mixed (1:1) with each dilution and the mixtures were allowed to react for 40 min at room temperature. The absorbance of the mixture was determined at 630 nm with a spectrophotometer. The dose–response data were plotted, but to obtain the curve equation, was used only linear range between 0.6 and 0.04 mol of EDTA disodium salt.

The siderophore concentration was expressed as equivalents to EDTA, calculated with the help of equation of EDTA concentration–absorbance. Data were subjected to analysis of variance in the  $2 \times 5$  factorial design (2 culture media amended or not with Fe<sup>3+</sup>, and 5 antagonists). The means were compared with the use of Tukey's test ( $p = 0.10$ ). This assay was done twice.

#### 2.7. Siderophores and germination of basidiospores of *M. pernicioso*

The supernatants used for siderophore quantification, were diluted serially with PBS buffer (100 mmol, pH 7.0). An aliquot of 20  $\mu$ L from each dilution was placed on water agar in a culture plate and mixed with 10  $\mu$ L of the basidiospore suspension. After 3 h incubation at 25 °C, basidiospore germination was evaluated as described previously. The control consisted of 20  $\mu$ L of the respective culture medium. Data were subjected to Pearson correlation analysis. To detect possible interference of the culture medium on basidiospore germination, data were also subjected to analysis of variance in the  $2 \times 5$  factorial design (2 culture media, with or without Fe<sup>3+</sup> and 5 antagonists). The means were compared with use of Tukey's test, ( $p = 0.10$ ).

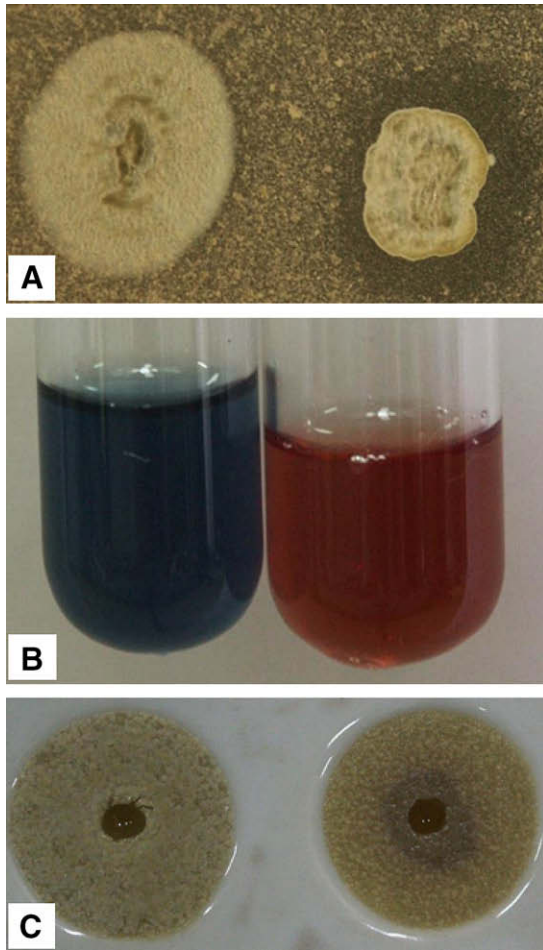
#### 2.8. Representation of data of germination inhibition

Germination inhibition by chitinase and siderophores was determined as described above. The basidiospore germination data for these tests are expressed as equivalents of the amount of Cu<sup>2+</sup> necessary to cause the same level of germination inhibition. The copper ion was selected as a standard fungicide because is an easily soluble in water and low dangerous to handle. Copper fungicides are used to control a witches' broom disease on Brazil (Souza et al., 2006). A dose–response curve for Cu<sup>2+</sup> concentration and basidiospore germination was plotted. Stock solutions of 0.91 g mL<sup>-1</sup> of Cu<sup>2+</sup> were prepared by dissolving 3.6 mg CuSO<sub>4</sub>·5H<sub>2</sub>O mL<sup>-1</sup> in distilled water and serially diluting the mixture to the same proportions by adding an aliquot of each dilution with an equal volume of the basidiospore suspension and placing the mixture on 1.5% water agar in culture plates. There were three replications for each treatment. Basidiospore germination was evaluated after 3 h incubation at 25 °C. For each repetitions of each treatment 50 spores were evaluated. The dose–response data were plotted, but to obtain the equation for the curve, only the values from the linear phase (22.9–0.72 g mL<sup>-1</sup> of Cu<sup>2+</sup>) were used to calculate Cu<sup>2+</sup> equivalence responsible for germination inhibition. This assay was done twice.

### 3. Results

#### 3.1. Production of lytic enzymes and inhibition of germination of basidiospores of *M. pernicioso*

All 5 of the isolates produced chitinase, as indicated by the appearance of a clear halo, around the colonies contrasting with the cloudy appearance of the unaffected medium (Fig. 1A). Culture plates covered for 90 min with 0.5% congo red solution



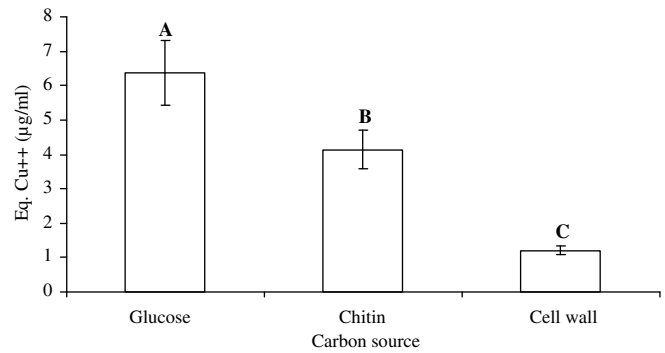
**Fig. 1.** Production of chitinases and hydroxamate type siderophores. (A) Chitinase producing isolates (right) with the clear halo around the colony contrasting with the turbid culture medium and the non-producer of chitinase (left). (B) Siderophore producing (right) and non-producing (left) isolates. (C) Isolate producing hydroxamate type siderophores are indicated by dark halo around the well (right) and non-producing isolate (left).

showed no pale-orange zones around the colonies, suggesting that  $\beta$ -1,3-glucanases or cellulases were produced with the carbon sources tested (Renwick et al., 1991). The carbon source did not affect enzyme activity and there were no significant differences among the isolates as to the quantity of chitinase produced (data not shown).

For all the antagonists, the inhibition of basidiospore germination was more pronounced when cultivated on glucose as carbon source, followed by colloidal chitin and cell wall preparation (Fig. 2 and 3). The antagonist, however, differed significantly in their capacity to inhibit basidiospore germination (Fig. 3).

### 3.2. Detection of siderophores and inhibition of basidiospore germination

The different antagonists produced only hydroxamate type siderophores (Fig. 1B). Siderophores were produced only in medium devoid of  $\text{Fe}^{3+}$  (Fig. 1B and C). Germination inhibition differed significantly among the supernatants sourced from different antagonists. Supernatants from cultures of *S. albovinaceus*, *S. griseus*, and *S. virginie* differed significantly (Tukey's test,  $p = 0.10$ ) in inhibiting germination depending upon  $\text{Fe}^{3+}$  supplementation, because the inhibition was more pronounced in culture medium without  $\text{Fe}^{3+}$  supplementation (Fig. 4). For this three isolates, probably sidero-



**Fig. 2.** Effect of carbon source on the production of substances inhibiting germination of basidiospores of *Moniliophthora perniciosa* present in the supernatants of the cultures of the antagonists on different carbon sources. Histograms with the same letter do not differ according to Tukey's test at  $p = 0.10$ .

phores is involved on inhibition spore germination. There were no significant differences among the antagonists as to siderophore production nor was there a correlation between the quantity of siderophore produced and the germination inhibition (data not shown).

## 4. Discussion

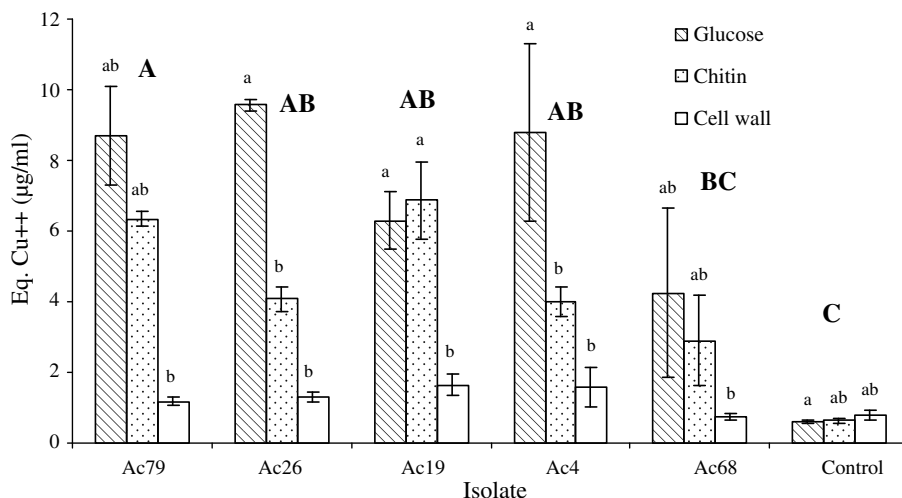
Chitinase production by actinomycetes is common, therefore Hsu and Lockwood (1975) developed a semi-selective culture medium for isolating this group of organisms, based on its constitutive production. In this study, no influence of carbon source on chitinase production was observed, and even in the absence of chitin considerable amount of chitinase was produced.

It is possible that chitinases have only an additive role in the antagonism of actinomycetes studied in this work, because the maximum germination inhibition occurred by the supernatants obtained from cultures grown on glucose, compared to chitin or cell walls as carbon source (Fig. 2). These results can be attributed to the greater availability of easily assimilable nutrients in the glucose containing medium, resulting in more abundant growth of the antagonists (data not shown), which suggests that the antagonism is more accentuated when the growth medium is more favorable to growth.

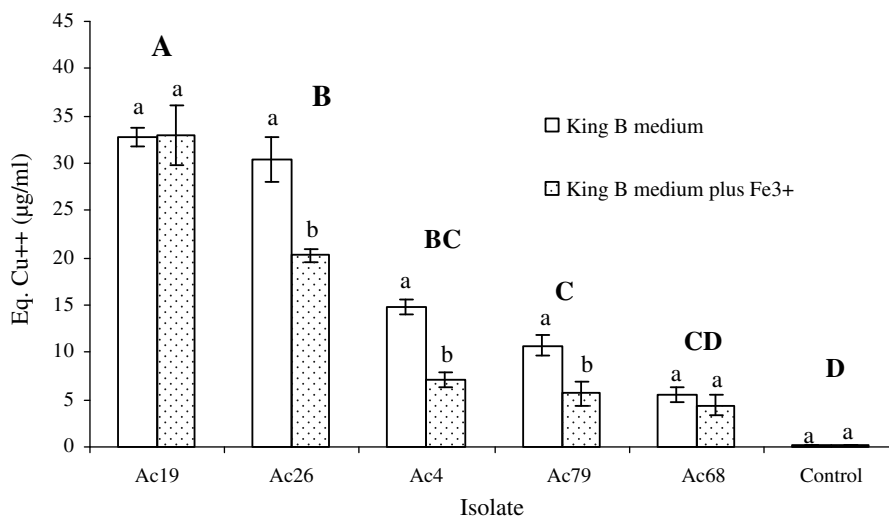
There is also the possibility of influence of the chitin isomers, since at least 20 isomers of this enzyme are known (Selitrennikoff, 2001). Roberts and Selitrennikoff (1988) noted that the endo-chitinases, generally produced by plants are more efficient in degrading intact fungal cell wall than the exo-chitinase produced by bacteria or actinomycetes. Data from this work suggest that chitinases produced by the actinomycetes belong to forms not effective on the basidiospores of *M. perniciosa*. However, De Boer et al. (1998) showed that chitinolytic microorganisms are better inhibitors when combined with other mechanisms of antagonism. It is notable that among the 332 isolates screened (Macagnan et al., 2006), the 5 best antagonists against *M. perniciosa* were all chitinolytic.

None of the 5 species of *Streptomyces* produced cellulases or  $\beta$ -1,3-glucanases, which are less common than chitinases (De Boer et al., 1998). According to Kaur et al. (2005), the simple availability of substrate, especially when supplied in purified form, is not sufficient to induce synthesis of these enzyme because some other factors may be required, which warrants additional studied under environments simulating the antagonist–pathogen interaction.

The lytic enzymes investigated in this work may be of less importance in antagonism, but since the chitinases are not substrate specific and also degrade other compounds including pro-



**Fig. 3.** Inhibition of germination of the basidiospores of *Moniliophthora perniciosa* by the supernatants of the cultures of the *Streptomyces virginiae* (Ac79), *S. griseus* (Ac26), *S. caviscabies* (Ac19), *S. albovinaceus* (Ac4), and *S. setonii* (Ac68) grown on glucose, chitin or cell walls preparation as carbon source. Capital letters compare the species and the small letters compare carbon sources. Histograms with the same letter do not differ (Tukey's test,  $p = 0.10$ ).



**Fig. 4.** Inhibition of germination of the basidiospores of *Moniliophthora perniciosa* by the supernatants of the cultures of the *Streptomyces virginiae* (Ac79), *S. griseus* (Ac26), *S. caviscabies* (Ac19), *S. albovinaceus* (Ac4), and *S. setonii* (Ac68) grown culture medium with or without amendment with  $Fe^{3+}$ . Capital letters compare the species and the small letters compare media with or without  $Fe^{3+}$  amendment. Histograms with the same letter do not differ (Tukey's test,  $p = 0.10$ ).

teins (Radwan et al., 1994) it may contribute to antagonistic activity through persistence in the environment. It is well known that the microbial antagonism is often attributable to a combination of mechanisms (Arora et al., 2008).

All the antagonists produced siderophores only in  $Fe^{3+}$  free medium. According to Neilands (1995) such compounds are produced only under conditions of low  $Fe^{3+}$  availability.

The production of siderophores appears to be involved in inhibiting basidiospore germination by the culture supernatants of *S. albovinaceus*, *S. griseus*, and *S. virginiae*, because the supernatants from  $Fe^{3+}$  deficient medium showed lower levels of germination compared those from  $Fe^{3+}$  supplemented medium (Fig. 4). The siderophores of these species appear to have high  $Fe^{3+}$  complexing capacity, depriving the pathogen of this nutrient. Similar results have reported for *Penicillium expansum* Link where the dormant conidia require iron for initiation of germination (Charlang et al., 1981). Although the basidiospores of *M. perniciosa* do not have constitutive dormancy, the siderophores produced by the antagonists, may have restricted iron availability, as reported by Calvente et al.

(1999) for conidia of *P. expansum* by the siderophores produced by a yeast.

The ability of different species of *Streptomyces* to inhibit germination of the basidiospores of *M. perniciosa* (Fig. 3) shows a pattern similar to that reported earlier on *T. cacao* fruit surfaces under field conditions (Macagnan et al., 2006). These high correlation of results showed *in vitro* with those obtained in the field are not very frequent in biological control studies with plant diseases.

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