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Short title: *Durability of efficacy of biological control*

Adaptation to pyrrolnitrin in *Botrytis cinerea* and cost of resistance

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Many biocontrol agents have been described for the control of *Botrytis cinerea*, but to date, scientific knowledge is scarce regarding the durability of their efficacy. The objective of our work was to estimate the risk of a decrease in the efficacy of biocontrol due to selection pressure exerted by biocontrol agents on *B. cinerea*. In this study, efforts have been focused on pyrrolnitrin, an antibiotic identified in diverse biocontrol agents having an effect on *B. cinerea*. To evaluate a possible decrease in sensitivity to pyrrolnitrin, ten successive generations of 5 isolates of *B. cinerea* were produced *in vitro* in the presence of a sub-lethal dose of the antibiotic (10µg L^{-1}). For one isolate, a significant reduction in the sensitivity to pyrrolnitrin at the 5th generation was observed with a resistance factor (RF) of ca. 11. The production of 10 additional generations for 4 of these isolates, with increasing doses of pyrrolnitrin (from 100 to 4000 µg L^{-1}), resulted in the development of variants of *B. cinerea* with high levels of resistance to the antibiotic (RF > 1000) and a reduced sensitivity *in vitro* to a pyrrolnitrin-producing bacterium (mean growth inhibition of 24 ± 7% for the pyrrolnitrin-resistant generations *vs*. 70 ± 3% for the sensitive parent isolates). Reverse adaptation of resistant variants after 10 additional generations in absence of selection pressure was not observed suggesting a stability of the resistance. Comparison of the pyrrolnitrin-resistant generations and their sensitive parent isolates for mycelial growth, sporulation and aggressiveness on plant tissues reveals that the high level of resistance to the pyrrolnitrin has resulted in a high fitness cost. Reduction in mycelial growth was about 1.7 to 3.6 times and reduction in sporulation was about 3.8 to 6.6 times. Aggressiveness was between 7 and 11 times lower on tomato and 3 to 10 times lower on apple. This study provides evidence that a fungal plant pathogen is able to gradually build-up resistance to an antibiotic produced by a biocontrol agent.

Keywords: biological control, durability, experimental evolution, fitness, resistance, selection

Introduction

Botrytis cinerea Pers.:Fr (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is the causal agent of grey mould, an economically important disease that generates losses on a wide range of crops (Jarvis, 1980). The control of this disease relies mainly on the use of fungicides but the development of resistance of *B. cinerea* populations to the different families of fungicides complicates their use and threatens their effectiveness (Leroux, 2004). Biological control could be a good alternative or complement and many biological control agents against *B. cinerea* have been described in the past years (Elad & Stewart, 2004, Elmer & Reglinski, 2006). The known modes of action include hyperparasitism, induction of plant-host resistance, competition for nutrients and the production of inhibitory metabolites (Elad & Stewart, 2004).

The antibiotic pyrrolnitrin (3-chloro-4-(2' -nitro-3' -chlorophenyl)-pyrrole) is produced by various bacteria such as *Enterobacter agglomerans* (Chernin *et al.*, 1996), *Serratia plymuthica* (Vleesschauwer & Hofte, 2007) and different species of *Pseudomonas* or related genera (de Souza & Raaijmakers, 2003) described as potential biological control agents against several plant pathogens. Several pyrrolnitrin-producing bacteria have been described as biological control agents against *B. cinerea* (Janisiewicz & Roitman, 1988, Chernin *et al.*, 1996). Moreover, the antibiotic itself has been reported to inhibit the growth of bacteria (El-Banna & Winkelmann, 1998) and fungi (Levenfors *et al.*, 2004) including *B. cinerea* (Janisiewicz & Roitman, 1988, Hammer *et al.*, 1993, Schoonbeek *et al.*, 2002). It has also received much attention in the medical field due to its activity against some opportunistic fungal or bacterial human pathogens including the causal agent of tuberculosis (Biava *et al.*, 2007).

Some recent studies have shown that *B. cinerea* can withstand a wide variety of fungitoxic compounds from different origins, including the principal families of fungicides (Leroux *et al.*, 2002), the grapevine phytoalexin resveratrol (Schoonbeek *et al.*, 2001) and the antibiotic 2,4 diacetylphloroglucinol (2,4 DAPG) produced by *Pseudomonas* spp. (Schouten *et al.*, 2008). Thus, *B. cinerea* appears to be a very adaptable fungus. The multinucleate and possibly heterokaryotic nature of this fungus, the presence of transposons in the genome, the existence of virus-like particles and the possible occurrence of sexual reproduction (Beever & Weeds, 2004) may explain this high level of variability. These known biological properties of *B. cinerea* might, potentially, compromise the durability of biological control methods despite the commonly reported assumption that resistance of plant pathogens or pests to biological control agents will develop less frequently as compared to chemical control methods (Holt & Hochberg, 1997, Duffy *et al.*, 2003). Although information on the durability of biological control efficiency is scant, recent results concerning pest management in agricultural systems have shown that this assumption may not always be justified (Asser-Kaiser *et al.*, 2007). Moreover, inconsistent efficacy of biological control against plant pathogens still appears to be frequent in the field (Elad & Stewart, 2004). This fact is generally attributed to variable climatic conditions encountered in the field, the lack of ecological competence of the biological control agent, and/or an unstable quality of the products (Elad & Stewart, 2004). However, a possible reduction of efficacy of a biological control agent could also arise if plant pathogens produce natural mutants with reduced susceptibility to the biological control agents used on crops. Knowledge on the potential development of resistance to biological control agents can thus help to devise or improve resistance management strategies. The build-up of field resistance to biological control agents could result from the selection of pre-existing plant pathogen isolates with low susceptibility in natural populations or it could arise if plant pathogens have the ability to produce natural mutants with reduced susceptibility under the selection pressure of products used by farmers.

The main purpose of the present study was to assess the potential for build-up of resistance in *B. cinerea* isolates to pyrrolnitrin-producing biological control agents. To this end,

successive generations were produced *in vitro* for five isolates of *B. cinerea*. Our specific objectives were (i) to monitor these generations for possible changes in sensitivity to pyrrolnitrin and to a pyrrolnitrin-producing biological control bacterium and in case of detection of resistant variants, (ii) to assess their fitness in comparison with the parent isolates and (iii) to evaluate the stability of the resistant phenotype by producing additional generations in absence of selection pressure.

Materials and methods

Isolates of *Botrytis cinerea*

Five single-spore isolates of *B. cinerea* originating from France were selected from a collection maintained in our laboratory. The choice was made on the basis of differences in aggressiveness to tomato plants and apple fruits and of differences in patterns of resistance to six fungicides representing four chemical groups. All selected isolates were sensitive to pyrrolnitrin (Table 1). For the duration of this work, they were maintained in stock cultures stored at -20°C in a 0.06 M phosphate buffer containing 20% (V/V) of glycerol.

In vitro conidial-transfer experiment

This experiment was carried out to produce 20 consecutive generations of spores on Potato Dextrose Agar medium (PDA 39 g L^{-1}; Difco Laboratory) amended with pyrrolnitrin (purified microbial pyrrolnitrin >95%, Sigma chemical). The pyrrolnitrin was dissolved in methanol and a stock solution containing 1 g L^{-1} was prepared and stored at 4°C in the dark to preserve its activity. Stored spore suspensions of the five isolates of *B. cinerea* were first inoculated on PDA medium without pyrrolnitrin. After 14 days of incubation at 21°C, spores were harvested from the plates in 2 mL of a phosphate-glycerol solution. This spore suspension represented the initial parental generation G0. To produce the next generation, this suspension was adjusted to 10^6 spores per mL, using a haemocytometer, and 40 µL aliquots were spread on Petri plates containing PDA medium amended with pyrrolnitrin at a final concentration of 10 µg L^{-1}. After 14 days of incubation in the dark at 21°C, spores representing generation G1 were harvested in 2 mL of a phosphate-glycerol solution. The spore suspension was adjusted to 10^6 spores per mL and 40 µL aliquots were used to produce the next generation. The remaining suspension was stored at –20°C. From G0, ten successive generations (G1 to G10) were produced on PDA medium in the presence of 10 µg L^{-1} of pyrrolnitrin (Fig. 1). This concentration of pyrrolnitrin was determined, in a preliminary experiment described below, as the highest dose allowing sufficient conidial production by any of the five isolates of *B. cinerea* after 14 days of incubation at 21°C in the dark. Using the spores of generation G10, ten additional successive generations were produced in conditions of increasing selection pressure. The concentration of pyrrolnitrin in the PDA medium was progressively increased from 10 µg L^{-1} (for the production of the first ten generations) to 4000 µg L^{-1} for the production of generation G20 (Fig. 1). Spores from alternate generations (G12, G14, G16, G18 and G20) were preserved at -20°C for further work. As a control, twenty successive generations were also produced on a pyrrolnitrin-free PDA medium.

For each of the five isolates, the whole experiment was carried out three times independently, providing three lineages of 20 generations produced under selection pressure (Fig. 1) and three independent "control" lineages produced on pyrrolnitrin-free PDA. To facilitate reading and avoid lengthy repetitions in the rest of this paper, control generations produced on unamended PDA and generations produced in presence of pyrrolnitrin will be labelled GnC and GnP, respectively, where n indicates the generation rank in the lineage.

Determination of pyrrolnitrin sensitivity

In a preliminary experiment, we assessed the effect of pyrrolnitrin on mycelial growth and spore production by the parental generation of each isolate. This information was needed to

determine the dose of pyrrolnitrin to be used in the production of successive generations under selective pressure as described above. In a second phase, sensitivity to pyrrolnitrin was tested on generations G1P, G2P, G3P, G4P, G5P, G7P, G10P, G12P and G20P for each of the 15 lineages generated under selection pressure. In addition, these tests were also conducted for generations G10C and G20C of the lineages produced on pyrrolnitrin-free medium for comparison.

The effect on mycelial growth was assessed on PDA medium containing different concentrations of pyrrolnitrin (0, 1, 2, 5, 10, 50, 100, 500, 1000, 2000, 4000, 6000, 8000 and 13000 µg L^{-1}). To carry out this test, the spores of a given generation (including parental generation G0) were first transferred to unamended PDA medium and the plates were incubated for four days at 21°C in the dark. Mycelial plugs (5 mm in diameter) were then excised from the periphery of the growing colonies and transferred to the centre of fresh PDA Petri plates (90 mm in diameter) containing the desired concentrations of pyrrolnitrin. The plates were incubated at 21°C in the dark and colony diameter was monitored for four days after inoculation. For each of the three lineages per isolate, three replicate plates were used for each dose of pyrrolnitrin and the whole experiment was carried out three times independently. The 50% effective concentration (EC_{50}) was estimated as described by Leroux *et al.* (1999). A resistance factor (RF) was measured as the ratio of the average EC_{50} value of a given generation for a given isolate (mean for the three replicate lineages) over the EC_{50} value of the sensitive parent of the considered isolate (generation G0). Statistical analyses were performed separately for each isolate on the EC_{50} values, to test the hypothesis that the level of sensitivity to pyrrolnitrin remained identical for the successive generations. In these analyses, we used the ANOVA module of Statistica software to test both a possible lineage effect and a generation effect. When a significant effect was observed, the multiple comparison test of Student-Newman-Keuls was used to compare the means.

The effect of pyrrolnitrin on spore production was assessed for the parental generation G0 of each isolate. Plates of PDA medium amended with different concentrations of pyrrolnitrin were inoculated in their centre with a mycelial plug (5 mm in diameter) taken from the periphery of a 4-day-old colony. After 14 days of incubation at 21°C in the dark, the spores were scraped from the medium, suspended in water and spore concentration was determined using a haemocytometer. Three replicate plates were inoculated for each dose of pyrrolnitrin and the whole experiment was carried out three times independently. The data were analysed with Statistica software, using the three repetitions of the experiment as blocks and the individual plates as replications.

Stability of pyrrolnitrin resistance

To test the possibility of "reverse adaptation" of resistant variants in absence of selection pressure, we studied the resistant variants obtained at the 20^{th} generation under selection pressure (G20P) for isolates BC1, BC25, BC26 and H6. For one lineage per isolate, spores of generation G20P were used to produce ten additional generations on PDA medium without pyrrolnitrin (G21 to G30). An incubation time of 14 days was applied between each successive generation. The EC_{50} and RF values of the last generation (G30) were determined for each isolate as described above.

Interaction between *B. cinerea* and a pyrrolnitrin-producing bacterium

The antagonistic activity of a pyrrolnitrin-producing bacterium was tested *in vitro* against the parental generation G0 of four isolates of *B. cinerea* (BC1, BC25, BC26 and H6) and against some of their generations obtained under selection pressure (G10P and G20P). For this work, one lineage was used per isolate. We used strain ChPhzS24 of *Pseudomonas chlororaphis* (Mazurier *et al.*, 2009), which was kindly provided by P. Lemanceau (INRA Dijon, France). Confrontation tests were conducted on TSA medium (3 g L^{-1} Tryptic Soy broth and 15 g L^{-1}

Agar) in 90 mm-diameter Petri plates. In each plate, the fungus (5 mm diameter mycelial plug) and the bacterium (20 µL droplet dosed at 10^7 CFU per mL) were placed 25 mm apart. In control plates, a droplet of sterile distilled water was used instead of the bacterial suspension. Three replicate plates were inoculated for each test and incubated at 21°C in the dark. The whole experiment was conducted three times independently. Mycelial growth (radius in mm) was measured daily for five days after inoculation. For each test, the inhibition of mycelial growth was assessed with an index computed as [1- (radius of mycelial growth on control plates / radius of mycelial growth in presence of the bacterium)] x100. Additionally, the width of the inhibition zone was measured 5 days after inoculation. The level of sensitivity to the bacterium between the pyrrolnitrin-sensitive parents (G0) and the generations (G10P and G20P) were compared using the ANOVA module of Statistica software. For a given isolate, the average of the inhibition of mycelial growth index and the width of the inhibition zone values of the three independent repetitions were used as elementary replicates. When a significant effect was observed, the multiple comparison test of Student-Newman-Keuls was used to compare the means.

Estimation of fitness cost *in vitro*

Different parameters of fitness were studied for the pyrrolnitrin-sensitive parental generation G0 and for several generations of isolates BC1, BC25, BC26 and H6 which presented reduced sensitivity to pyrrolnitrin. These fitness parameters were assessed on PDA medium and included spore germination, mycelial growth and spore production. Spore germination was first assessed after 24 hours of incubation. It was estimated by examining 100 arbitrarily-selected spores from each of three replicate plates. As all tests systematically resulted in the observation of 100% germination, an attempt was made to explore possible differences in the early stage of the germination process. To this end, germination was also quantified every hour during a six-hour incubation period at 21°C. The early kinetics of germination were assessed by computing an average hourly rate of germination for the period between the 3rd and the 6th hour after inoculation. Mycelial growth and spore production were assessed as described above for the determination of pyrrolnitrin sensitivity, except that plates were placed in a growth chamber with a photoperiod of 14 hours to hasten sporulation. Mycelial growth was assessed by measuring the diameter of colonies 3 days after inoculation and by estimating the daily radial growth rate between 1 and 4 days after inoculation. Sporulation was computed as spores produced per Petri plate, 14 days after inoculation. The experiments were repeated three times, each with three replicates. Statistical analyses were performed separately for each isolate and each type of fitness parameter, to test the hypothesis that the fitness remained identical for the successive generations. In these analyses, we used the ANOVA module of Statistica software to test both a possible lineage effect and a generation effect. When a significant effect was observed, the multiple comparison test of Student-Newman-Keuls was used to compare the means.

Estimation of fitness cost *in planta*

To assess possible changes in aggressiveness, several generations were tested on apple fruits and tomato plants. For each generation of a given isolate, the aggressiveness of the three lineages was tested.

The aggressiveness of generations G0, G12P and G20P obtained with isolates BC1, BC25, BC26 and H6 was assessed on apple fruits cv. Golden. Generation G20C, produced on pyrrolnitrin-free medium, was included in all tests for comparison. In addition, generation G10P of BC26 was also tested because of its resistance to pyrrolnitrin. In these tests, three 2 mm-wounds were inflicted on the surface of the fruit and immediately inoculated with 10 µL of a spore suspension of *B. cinerea* containing 10^6 spores per mL. Apples were incubated in a

growth chamber at 21°C. The diameter of lesions was monitored daily for 7 days after inoculation.

Tests on tomato plants cv. Monalbo (INRA, Avignon, France) were conducted for generations G0, G12P and G20P of isolate BC1 and for generations G0, G10P, G12P and G20P of isolate BC26. Generation G20C, produced on pyrrolnitrin-free medium, was included in all tests for comparison. Two types of bioassays were used with two independent sets of plants. To test the aggressiveness of *B. cinerea* on the stem, leaves were removed from the plants leaving a 5 mm petiole stub on the stems and 10 µL of spore suspension were deposited on the petiole stub. Because isolate BC26 was known to have a low level of aggressiveness on tomato plants (Table 1), we used a spore concentration of 10^6 spores per mL for isolate BC1 and of 10^7 spores per mL for BC26. The lengths of resulting stem lesions were monitored daily for 7 days after inoculation. The aggressiveness of generations G0, G10P, G12P, G20P and G20C of isolate BC26 was also tested on petioles. To this end, leaves were removed from the plants leaving a 50 mm petiole stub and 10 µL of spore suspension containing 10^7 spores per mL were deposited on the wound. The length of lesions on petiole stubs was monitored daily for 7 days after inoculation. All plants were incubated in a growth chamber with a photoperiod of 14 hours and maintained at 21°C with a relative humidity above 90%.

The experiments were all repeated three times independently per lineage, each with three replicate plants or fruits. To take into account the kinetics of disease development for each isolate, we computed the AUDPC as described by Decognet *et al.* (2009). Statistical analyses were performed separately for each isolate on the AUDPC values, to test the hypothesis that the level of aggressiveness remained identical for the different generations. In these analyses, we used the ANOVA module of Statistica software to test both a possible lineage effect and a generation effect. When a significant effect was observed, the multiple comparison test of Student-Newman-Keuls was used to compare the means.

Results

Sensitivity to pyrrolnitrin of the wild-type parent isolates

The mycelial growth of the five isolates used in this study (parental generation G0) was highly affected by pyrrolnitrin, with estimated values of EC_{50} comprised between 1 and 7 µg L^{-1} (Table 2). At 10 µg L^{-1} pyrrolnitrin, the inhibition of mycelial growth ranged between 56.3% and 98.5% (respectively 64.8%, 56.3%, 81.9%, 71.9% and 98.5% for BC1, BC21, BC25, BC26 and H6). For a dose of pyrrolnitrin of 100 µg L^{-1} or greater, the mycelial growth of all five isolates was completely inhibited (Fig. 2).

Spore production of the five isolates was also significantly affected by pyrrolnitrin (ANOVA, $P = 0.009$ for BC1, $P = 0.011$ for BC21, $P = 0.039$ for BC25, $P < 0.0001$ for BC26 and $P < 0.0001$ for H6) (Fig. 2). At 50 µg L^{-1} pyrrolnitrin, sporulation was completely inhibited for three of the isolates and for higher concentrations the inhibition was complete for all isolates (Fig. 2). The dose of 10 µg L^{-1} pyrrolnitrin resulted in a reduction of spore production of 93.9±1.3% (average for the five isolates ± standard error) compared to the control without pyrrolnitrin. It allowed the production of spores in a range from $2.6x10^5$ to $9.0x10^6$ spores per 90 mm-diameter Petri plate depending on the isolate (Fig. 2). This dose of pyrrolnitrin was thus selected for the production of successive generations under selection pressure.

Sensitivity to pyrrolnitrin of successive generations produced under selection pressure

For all isolates tested, there was no significant change in the level of resistance to pyrrolnitrin for the control generations G10C and G20C produced in absence of pyrrolnitrin (ANOVA, $P > 0.05$). In contrast, changes in sensitivity were observed among the successive generations produced on PDA medium amended with pyrrolnitrin. The patterns of change were different

among the five isolates. For isolate BC26, sensitivity to pyrrolnitrin was significantly reduced after only four successive generations (Fig. 3). The phenomenon was identical for each of the three independent replicate lineages of this isolate and no significant differences among these lineages were observed in any of the sensitivity tests conducted for generations G4P, G5P, G7P or G10P (ANOVA, $P > 0.05$). The estimated EC_{50} of this isolate remained similar between G5P and G10P, with a corresponding resistance factor of *ca.* 11 (Fig. 3, Table 2). For isolates BC1, BC25 and H6, the level of resistance was almost unchanged after 10 generations on 10 µg L^{-1} pyrrolnitrin (Fig. 3, Table 2). In contrast, the sporulation of all three replicate lineages of isolate BC21 was so reduced after 7 generations on pyrrolnitrin-amended medium that it was impossible to produce a generation G8P with this isolate (Fig. 3). Work was thus pursued with only isolates BC1, BC25, BC26 and H6.

For these four isolates, spores from the 10th generation (G10P) were transferred to fresh PDA containing a higher concentration of pyrrolnitrin (Fig. 1). All replicate lineages for each isolate were able to grow and sporulate on 100 µg L^{-1} pyrrolnitrin, producing an 11th generation (G11P). This contrasted with the total inhibition observed with such a concentration for the parental generation G0 of all five isolates (Fig. 2). However, to obtain a sufficient number of spores for the production of the next generation, the incubation of generation G11P was extended from 14 to 30 days (Fig. 1). Following this step, the next generations (G12P to G20P) were all obtained after 14 days of incubation despite the gradual increase in the concentration of pyrrolnitrin (Fig. 1). After a total of 20 successive generations, the last 10 of which produced on increasing doses of pyrrolnitrin, high levels of resistance were obtained and the resistance factors were greater than 1000 for the 4 isolates (Table 2). As indicated by the small standard errors, similar values were obtained for the three lineages of each isolate (Table 2). The resistance factor did not increase from the 12th to the 20th generation (Fig. 3). This pattern of change in the resistance was similar for the four isolates even if they differed significantly in the maximum EC_{50} values achieved after 12 generations (ANOVA, $P = 0.005$, Table 2).

Stability of pyrrolnitrin-resistant variants

Using pyrrolnitrin-resistant generation G20P to produce ten additional generations on pyrrolnitrin-free medium did not lead to any decrease in sensitivity to the antibiotic for any of the isolates. In all cases, there was no significant difference between the EC_{50} values for generation G20P and G30 (Table 2).

***In vitro* interaction with pyrrolnitrin-producing bacteria**

In vitro confrontations of pyrrolnitrin-producing strain ChPhzS24 of *P. chlororaphis* with parental generation G0 of any of the isolates resulted in a measurable inhibition zone and substantial growth inhibition of *B. cinerea* (Fig. 4; Table 3). Inhibition also occurred with generation G20P, but for all isolates it was significantly reduced in comparison with G0. For generations G10P, there was no significant difference in inhibition by *P. chlororaphis* in comparison with generation G0 even of isolate BC26 which showed moderate resistance to pyrrolnitrin (Table 3).

***In vitro* fitness cost for pyrrolnitrin-resistant variants**

No significant differences were observed between the pyrrolnitrin-sensitive parents G0 and the resistant generations G20P for germination rate on PDA medium (ANOVA, $P > 0.05$, Table 4). There was no significant lineage effect for mycelial growth and sporulation on PDA medium of any of the isolates ($P > 0.05$). For every isolate on the other hand, the mycelial growth of the resistant variants G12P and G20P was significantly reduced ($P < 0.002$) compared with the pyrrolnitrin-sensitive parent G0 and the control generation G20C (Table 4, Fig. 4A and B). Spore production of the pyrrolnitrin–resistant variants G12P and G20P was

also significantly reduced ($P < 0.009$) for every isolate compared with the pyrrolnitrin-sensitive parents G0 and the control generation G20C (Table 4).

Level of aggressiveness of pyrrolnitrin-resistant variants on plant tissues

There was no significant lineage effect for the aggressiveness of any of the isolates ($P > 0.05$). For each of the four isolates tested, the pyrrolnitrin-resistant variants G12P and G20P were significantly less aggressive on apple fruits than the parental generation G0 and control generation G20C (Table 4). On tomato plants, a decrease of aggressiveness was also observed with generations G12P and G20P of isolates BC1 and BC26 compared with the parental generations G0 (Table 4).

Discussion

The present study provides for the first time evidence that a fungal plant pathogen is able to gradually build-up resistance to an antibiotic produced by a biological control agent. A high level of resistance to pyrrolnitrin appeared when *B. cinerea* was continuously exposed to the antibiotic pyrrolnitrin, indicating its capacity of adaptation in laboratory conditions. This adaptation was observed rapidly after the 10^{th} generation following an increase in the concentration of pyrrolnitrin used in the medium. It was observed for all the isolates tested regardless of their original phenotypic differences, suggesting an absence of isolate effect in the build-up of resistance. It was not possible to assess *in planta* the efficacy of biocontrol by the pyrrolnitrin-producing bacterium *P. chlororaphis* ChPhzS24 against the pyrrolnitrin-resistant generations G20P, due to the loss of aggressiveness of these generations. Nevertheless, *in vitro* tests revealed that the resistance of *B. cinerea* to pyrrolnitrin was associated with a decrease in the antagonistic effect of the bacterium.

However, the small remaining inhibition zones observed around the bacterial colonies in confrontation tests suggest that the pyrrolnitrin-resistant variants were not completely resistant to the bacterium. A possible explanation for this phenomenon may be related to the concentration of pyrrolnitrin present in the vicinity of the bacterium in the medium. Although the quantity of pyrrolnitrin produced by strain ChPhzS24 of *P. chlororaphis* on PDA medium is not known, one may hypothesise that resulting concentrations could be greater than those used in our tests to determine the level of resistance of *B. cinerea* to pyrrolnitrin *in vitro*. Another explanation could be that the variants of *B. cinerea* obtained on pyrrolnitrin-amended medium were probably not resistant to another antibiotic, phenazine-1-carboxylic acid, possibly also produced by strain ChPhzS24 of *P. chlororaphis* (Schoonbeek *et al.*, 2002).

Spore production in the presence of a high concentration of pyrrolnitrin (100 µg L^{-1}) was possible for the G10 variants of four isolates whereas it was not possible for the sensitive parental generation G0. This suggests that isolates already exposed to pyrrolnitrin may be better prepared to adapt to pyrrolnitrin-rich environments compared to isolates that have never been exposed to this antibiotic. Luo & Schnabel (2008) already proposed that the various levels of resistance to fungicides generated for different isolates of *Monilinia fructicola* may be linked to their original frequency of exposure to the fungicides under study. Therefore, this fungus may have reacted to its selective environment by accumulating some adaptive mutations, which may not necessarily include mutations that have a direct consequence on the resistance to the antibiotic (Rosenberg, 2001).

Previous studies have shown that the resistance of a fungus to a fungicide may predispose the resistance to other fungicides and evidence of such predisposition has been documented for *Monilinia fructicola* (Luo & Schnabel, 2008) and *Venturia inaequalis* (Koeller & Wilcox, 2001). This study does not show a similar phenomenon for the resistance of *B. cinerea* to pyrrolnitrin. When *B. cinerea* isolates were subjected to a stable and relatively low dose of

pyrrolnitrin before the 10th generation (10 µg L^{-1}), susceptibility to the antibiotic significantly evolved only for one of the five isolates tested (BC26). For this isolate, no evidence of predisposition was established as its fungicide-resistance pattern was not different from that of another isolate tested (BC1). Moreover, the speed of selection by pyrrolnitrin was not accelerated for the 4 other isolates tested whatever their pattern of fungicides resistance. Therefore, it appears that the development of pyrrolnitrin resistance in *B. cinerea* is independent of its original profile of resistance to fungicides.

Fitness is a key parameter for the build-up of resistance in the field. In this study, the resistance of *B. cinerea* variants to pyrrolnitrin was systematically associated with reduced growth potential (mycelial growth and spore production) and decreased aggressiveness on plants or fruits (reduction of lesion growth). This fitness cost was similarly high for the four isolates tested. Based on these results, we postulate that traits involved in resistance may be genetically linked to fitness cost and work is in progress to test this hypothesis. One practical consequence of this phenomenon is that if pyrrolnitrin resistance occurred in the field, the associated detrimental effect on the variant and competition from pyrrolnitrin-sensitive strains would limit the risk of complete loss of efficacy of pyrrolnitrin-producing biological control agents.

Several experimental studies have evaluated the relationship between fitness and fungicide resistance for *B. cinerea*, but results are quite contradictory to each other (Vignutelli *et al.*, 2002, Moyano *et al.*, 2004, Ziogas *et al.*, 2005, Bardas *et al.*, 2008). Bardas *et al.* (2008) showed for instance that the resistance of *B. cinerea* to the anilinopyrimidine fungicides did not affect their fitness compared to the sensitive isolates. However, our results are consistent with results reported by Ziogas *et al.* (2005) concerning the resistance of *B. cinerea* to fludioxonil, a fungicide whose structure is close to that of pyrrolnitrin (Leroux *et al.*, 2002). A comparison of fitness among wild-type isolates and fludioxonil-resistant mutants of *B. cinerea* showed that all fludioxonil-resistant mutants had significant reductions in the characteristics that determine saprophytic fitness, such as mycelial growth, sporulation, conidial germination and sclerotial production (Ziogas *et al.*, 2005). According to these authors, the polygenic control of resistance to fludioxonil may be responsible for the reduced fitness of fludioxonil-resistant isolates of *B. cinerea* obtained in the laboratory (Ziogas *et al.*, 2005). Concerning biological control, experimental evidence of fitness costs associated with resistance has only been reported for pests. For instance, selection experiments have shown that resistance of *Drosophila melanogaster* to parasitoids imposes fitness costs (Kraaijeveld & Godfray, 1997). To our knowledge, the present study is the first report of such a phenomenon for a plant pathogen.

The extent to which an organism is selected in a population depends on the advantages that the resistance provides but also on the costs of maintaining the resistance in the population in the absence of selection pressure. Ziogas *et al.* (2005) showed that fludioxonil-resistant variants of *B. cinerea* reverted to decreased resistance when they were grown in absence of the fungicide. In contrast in the present study, the level of resistance to pyrrolnitrin remained stable for 10 successive generations produced on pyrrolnitrin-free medium. This trait may constitute a risk factor in the field, if subsequent mutations could improve the fitness of the resistant variants as observed for antibiotic-resistant bacterial mutants (Andersson & Levin, 1999). Furthermore, the genetic recombination associated with sexual reproduction may produce new phenotypes in the population. Population genetics studies have revealed high levels of variability in *B. cinerea* and a lack of linkage disequilibrium within populations from different countries, suggesting that this fungus may undergo sexual reproduction (Giraud *et al.*, 1997, Karchani-Balma *et al.*, 2008, Vaczy *et al.*, 2008).

In practical terms, the development of reduced susceptibility to pyrrolnitrin in *B. cinerea* populations, if it should occur, may not necessarily jeopardize the efficacy of pyrrolnitrin-producing biocontrol agent. However, loss of efficacy of a biological control agent was already described for *B. cinerea*. Li & Leifert (1994) have shown that after 10 successive treatments on plants with the antibiotic-producing bacterium *Bacillus subtilis*, its efficacy against *B. cinerea* dropped dramatically. But the biological control activity of a given microorganism may be associated with other mechanisms in addition to antibiosis, and it was suggested that many biological control agents suppress disease by using more than one mode of action (Elad & Stewart, 2004). Furthermore, it was suggested that resistance to complex or multiple antagonistic traits should occur only at a low frequency (Duffy *et al.*, 2003).,,In future studies on biological control, nevertheless, it may be prudent to include work on the durability of efficacy of the biological control agents having multiple modes of action.

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Table 1 Characteristics, level of aggressiveness on tomato plants and on apple fruits, sensitivity to pyrrolnitrin and fungicides of *Botrytis cinerea* isolates used for the *in vitro* conidial-transfer experiments.

Isolate of *Botrytis cinerea*	Host	Year isolated	Aggressiveness[a]		pyrrolnitrin	Active ingredients of fungicides (chemical family)[b]			
			tomato plant	apple		Carbendazim (Benzimidazoles)	Iprodione, procymidone , vinclozolin (Dicarboximides)	fludioxonil (Phenylpyrroles)	dicloran (Aromatic Hydrocarbons)
BC1	tomato	1989	+++	+++	S[c]	S	R	S	R
BC21	strawberry	1991	++	+++	S	S	S	S	S
BC25	tomato	1991	-	++	S	S	S	S	S
BC26	tomato	1991	+	++	S	S	R	S	R
H6	tomato	1991	-	+	S	R	S	S	S

[a] +++ = high level of aggressiveness, ++ = moderate level of aggressiveness, + = low level of aggressiveness, - = not aggressive in our conditions of test

[b] the concentrations of fungicides used are as follows: carbendazim 1 µg mL^{-1}, Iprodione 2.5 µg mL^{-1}, procymidone 10 µg mL^{-1}, vinclozolin 5 µg mL^{-1}, fludioxonil 0.1 µg mL^{-1}, dicloran 25 µg mL^{-1}

[c] S = sensitive and R = resistant.

Table 2 Level and stability of resistance to pyrrolnitrin of the different generations produced from the five isolates of *Botrytis cinerea* BC1, BC21, BC25, BC26 and H6. The generations G0, G7P, G10P, G12P, and G20P are respectively the wild-type parent isolates, the 7th, the 10th, the 12th and the 20th generation produced in presence of pyrrolnitrin in the medium. For BC21, generations were not pursued after the 7th generation. The generation G30 is the 30th generation obtained from G20 by producing 10 consecutive generations on PDA medium without pyrrolnitrin.

Isolate of *Botrytis cinerea*	Generation tested	EC_{50} ($\mu g.L^{-1}$)[a]	RF[b]
BC1	G0	5.1 ± 0.1	
	G10P	9 ± 1	1.7
	G12P	8700 ± 1100	1700
	G20P	8700 ± 1100	1700
	G30	8700 ± 1100	1700
BC21	G0	7 ± 1	
	G7P	7 ± 1	1
BC25	G0	5 ± 2	
	G10P	9 ± 1	1.8
	G12P	9000 ± 600	1800
	G20P	9000 ± 600	1800
	G30	9000 ± 700	1800
BC26	G0	6 ± 1	
	G10P	63 ± 13	11
	G12P	7200 ± 500	1200
	G20P	7400 ± 400	1230
	G30	7400 ± 500	1230
H6	G0	1.3 ± 0.3	
	G10P	7 ± 1	5.4
	G12P	4600 ± 100	3540
	G20P	4600 ± 100	3540
	G30	4600 ± 200	3540

[a] average values obtained with three replicate lineages ± standard error of the mean

[b] RF = resistance factor calculated by dividing the EC_{50} value of each of the generations tested by the EC_{50} value of the sensitive parent of the considered isolate (G0)

Table 3 *In vitro* sensitivity of the pyrrolnitrin-sensitive and resistant variants of *Botrytis cinerea* to the pyrrolnitrin-producing bacterial strain *Pseudomonas chlororaphis* ChPhzS24. G0, G10P and G20P are respectively the wild-type parent isolates, the 10th generation and the 20th generation obtained in presence of pyrrolnitrin in the medium. Inhibition of mycelial growth index and width of the inhibition zone were estimated 5 days after inoculation. Data are means of three independent tests.

Isolate of *Botrytis cinerea*	Generation tested	Pyrrolnitrin (RF)[a]	Effect of *Pseudomonas chlororaphis* ChPhzS24			
			Growth inhibition (%)[b]		Inhibition zone (mm)	
BC1	G0		74.8	a	18.3	a
	G10P	1.7	72.6	a	19.3	a
	G20P	1700	36.5	b	7.0	b
BC25	G0		66.7	a	19.5	a
	G10P	1.8	63.7	a	20.0	a
	G20P	1800	6.5	b	7.5	b
BC26	G0		74.6	a	16.6	a
	G10P	11	69.2	a	13.0	ab
	G20P	1230	17.9	b	8.5	b
H6	G0		64.4	a	23.2	a
	G10P	5.4	58.9	a	20.9	a
	G20P	3540	34.3	b	3.5	b

[a] RF= resistance factor calculated by dividing the EC_{50} value of each of the generations tested by the EC_{50} value of the sensitive parent of the considered isolate (G0). For each isolate, means within a column followed by the same letter are not significantly different (ANOVA, $\alpha = 0.05$; Newman–Keuls test).

[b] Growth inhibition = [1- (radius of mycelial growth on control plates in mm / radius of mycelial growth in presence of the bacteria in mm)] x100

Table 4 Comparison of fitness between pyrrolnitrin-resistant generations, control generations and the wild-type parent isolates G0. The different components of fitness evaluated are: hourly germination rate between the 3rd and 6th hour of incubation (% per hour), colony diameter 3 days after inoculation (mm), daily radial growth rate between the first and 4th day after inoculation (mm per day), spore production on PDA medium 14 days after inoculation (number of spores produced per Petri plate) and aggressiveness on tomato plant and apple fruits.

Isolate of *Botrytis cinerea*	Generation tested	Hourly germination rate[a]	Mycelium[a]		Sporulation[a]	Aggressiveness (AUDPC)[a]		
			colony diameter	daily radial growth	x10^6 spores per plate	tomato plant		apple
		% per hr	mm	mm/day		petiole stub	stem	
BC1	G0[b]	15.0 a	76.3 a	23.0 a	156 a	-	82.2 a	184.1 a
	G20C	-[c]	79.0 a	25.1 a	157 a	-	68.3 a	139.0 a
	G12P	-	38.3 b	12.0 b	66 b	-	10.3 b	20.0 b
	G20P	16.5 a	36.7 b	12.4 b	40 b	-	7.3 b	19.3 b
BC25	G0	12.3 a	67.3 a	22.3 a	130 a	-	-	126.7 a
	G20C	-	66.7 a	23.4 a	98 a	-	-	124.1 a
	G12P	-	41.0 b	13.5 b	47 b	-	-	54.3 b
	G20P	9.2 a	40.3 b	14.6 b	34 b	-	-	49.1 b
BC26	G0	22.0 a	58.5 a	23.6 a	155 a	66.6 a	10.4 a	114.0 a
	G20C	-	55.7 a	23.7 a	66 b	83.1 a	16.5 a	111.1 a
	G10P	-	56.5 a	21.0 a	-	87.4 a	9.0 a	97.1 b
	G12P	-	38.0 b	14.0 b	32 c	-	-	24.7 c
	G20P	19.7 a	35.0 b	14.2 b	32 c	9.1 b	0.0 b	19.3 c
H6	G0	20.0 a	54.3 a	19.7 a	105 a	-	-	89.5 a
	G20C	-	50.3 a	18.3 a	92 a	-	-	94.3 a
	G12P	-	18.0 b	6.0 b	46 b	-	-	28.7 b
	G20P	17.3 a	15.0 b	6.7 b	16 c	-	-	22.0 B

[a] Data are means of the three lineages. For each isolate, means within a column followed by the same letter were not significantly different (ANOVA, $\alpha = 0.05$; Newman–Keuls test).

[b] G0 is the wild-type parent isolate, G20C is the 20th generation produced on PDA medium without pyrrolnitrin (control). G10P, G12P and G20P are respectively the 10th, the 12th and the 20th generation produced on PDA amended with pyrrolnitrin (10 µg L^{-1}, 100 µg L^{-1} and 4000 µg L^{-1}, respectively).

[c] - = not done or not relevant

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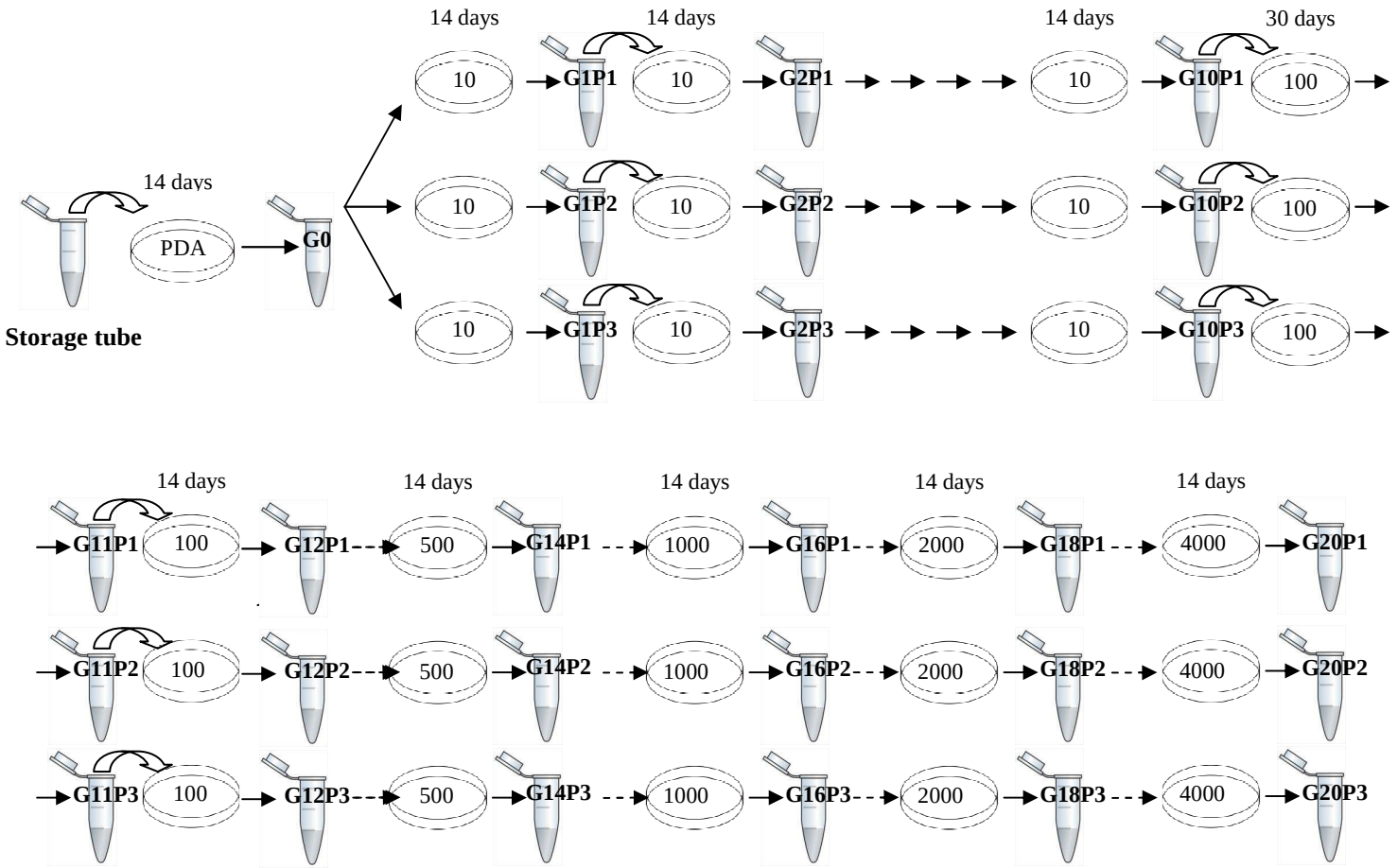

Figure 1 Presentation of the conidial-transfer procedure for *Botrytis cinerea* isolates. The concentration of pyrrolnitrine in µg L^{-1} is indicated in the plates for every generation. For the pyrrolnitrin generations, the PDA medium was amended with 10 µg L^{-1} of pyrrolnitrin until the 10^{th} generation, then with increasing doses of pyrrolnitrin to reach 4000 µg L^{-1} at the 20^{th} generation. Control generations were produced on PDA medium without pyrrolnitrin. Three independent conidial-transfer procedures (lineages 1, 2 and 3) were realised for each isolate. Every generation was transferred to a new plate every 14 days except the 11th generation which was incubated for 30 days.

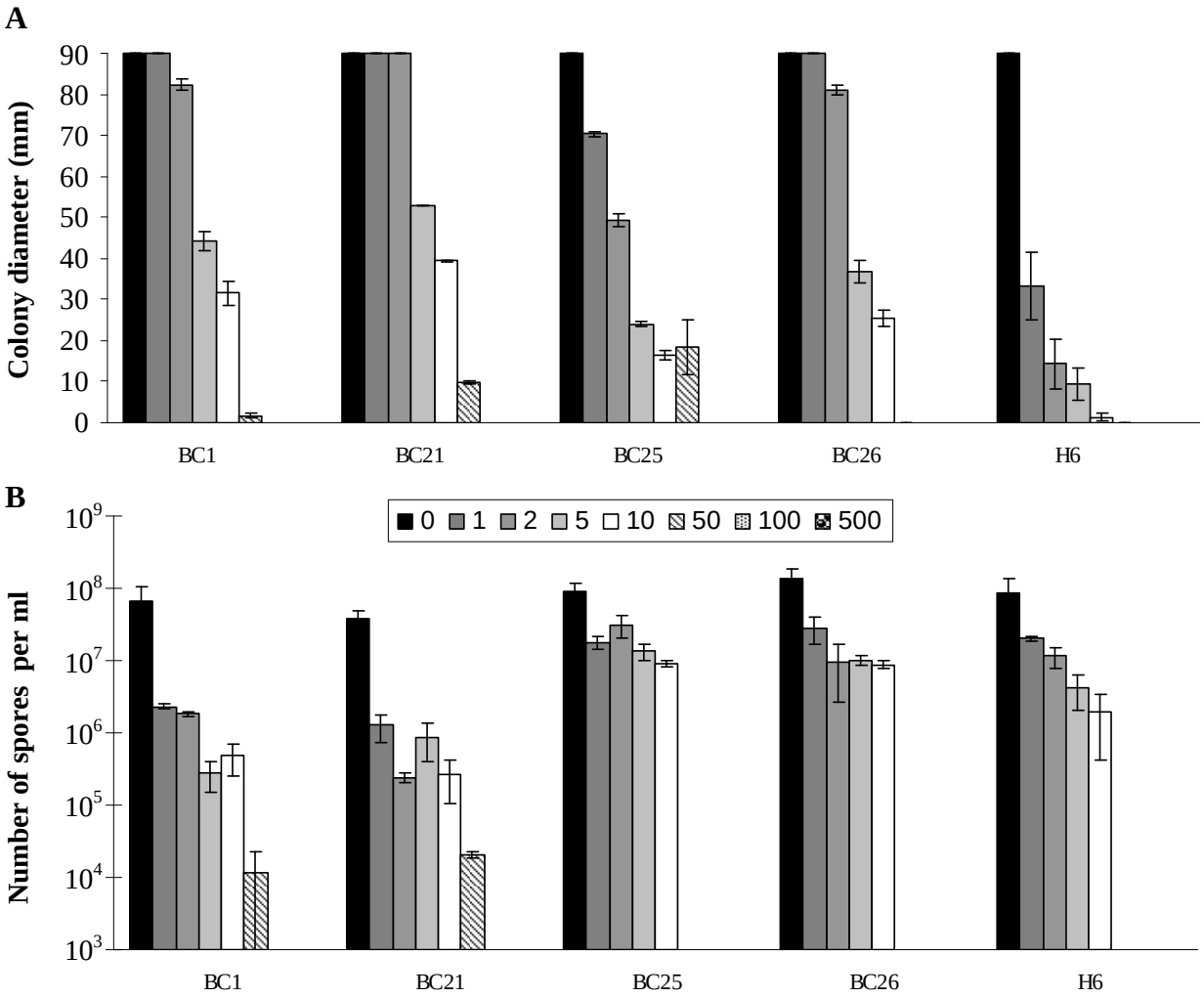

Figure 2 Colony diameter (A) and spore production (B) estimated respectively 4 and 14 days after inoculation, for parental generation G0 of five *Botrytis cinerea* isolates on PDA medium amended with different concentrations of pyrrolnitrin (in µg L^{-1}). Each bar represents the average value for three independent repetitions. Error bars indicate the standard error of the mean.

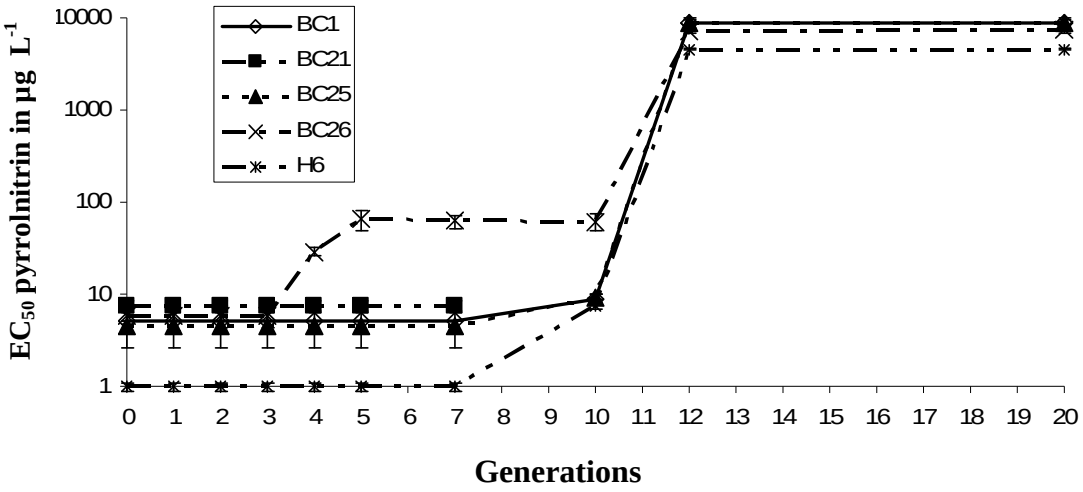

Figure 3 Evolution of resistance to pyrrolnitrin for 5 isolates of *Botrytis cinerea* maintained for 20 successive generations on PDA amended with pyrrolnitrine. From parental generation G0, ten successive generations (G1P to G10P) were produced in the presence of 10 µg L^{-1} of pyrrolnitrin. Using the spores of generation G10, ten additional successive generations were produced in conditions of increasing selection pressure. The concentration of pyrrolnitrin in the PDA medium was progressively increased from 100 µg L^{-1} for the production of the 11th generation to 4000 µg L^{-1} for the production of generation G20. The 50% effective concentration (EC_{50}) was estimated for the generations G0 - G5, G7, G10, G12 and G20. Values of EC_{50} correspond to the average of the 3 independent lineages and error bars indicate the standard error of the mean.

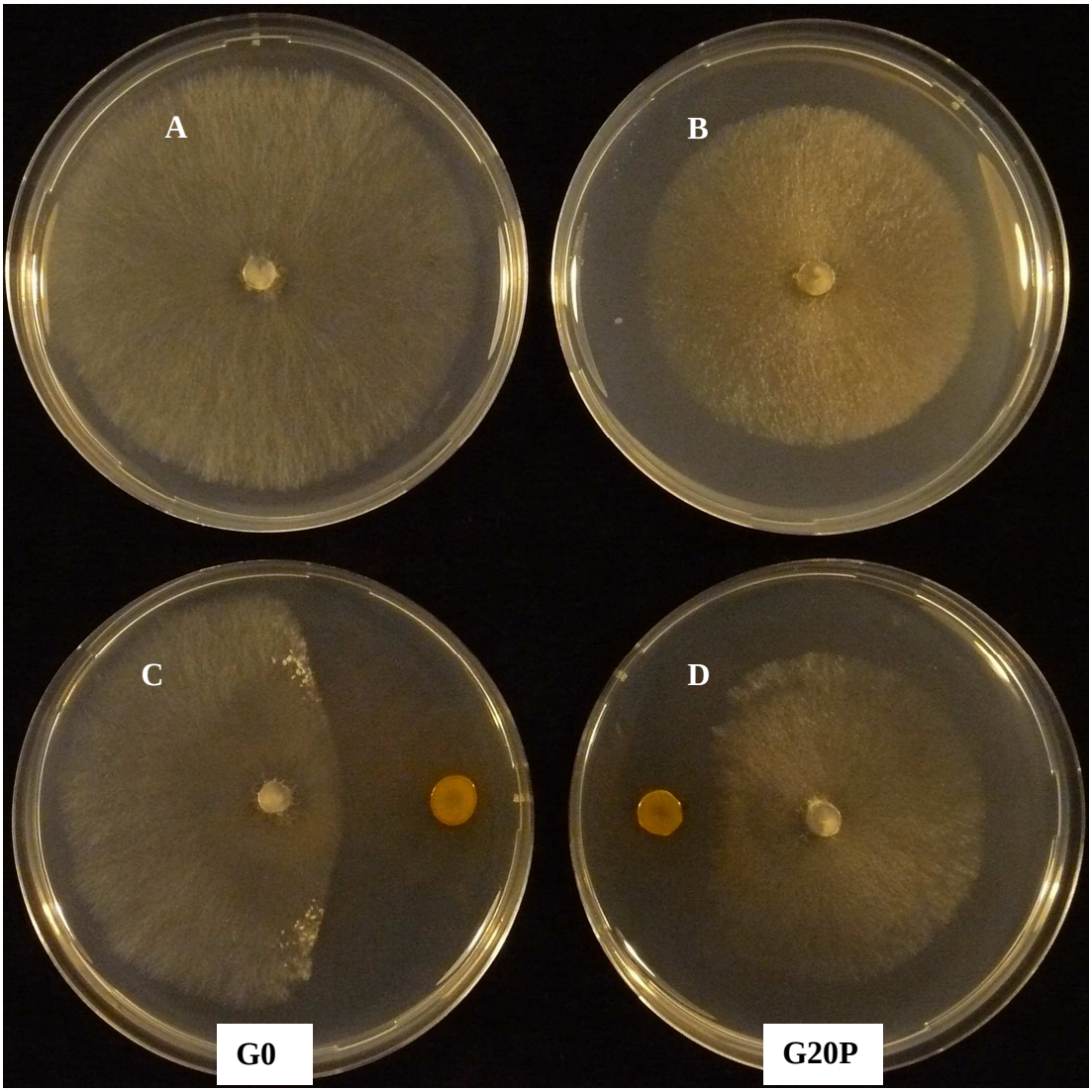

Figure 4 *In vitro* sensitivity of *Botrytis cinerea* isolate BC1 to the pyrrolnitrin-producing *Pseudomonas chlororaphis* strain ChPhzS24. The pyrrolnitrin-sensitive parent isolate (G0) and the pyrrolnitrin-resistant variant (G20P) were compared 5 days after inoculation. Control plates A and B show mycelial growth of G0 and G20P on TSA medium without the bacterium. The effect of the bacterium on G0 and G20P is exemplified by plates C and D.