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ORIGINAL ARTICLE

Brazilian isolates of *Clonostachys rosea*: colonization under different temperature and moisture conditions and temporal dynamics on strawberry leaves

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Abstract

Aims: In a research programme for managing diseases caused by *Botryis cinerea*, four isolates of the antagonistic fungus *Clonostachys rosea* (*Cr*) were obtained from different ecosystems in Brazil. We studied ecological requirements for the colonization of strawberry leaves by these isolates.

Methods and Results: Temperature effects on both mycelial growth *in vitro* and leaf colonization by *Cr* were studied. At 10°C, growth on potato dextrose agar and colonization of leaf discs were poor. Optimum temperature for mycelial growth and leaf colonization was around 25°C. The isolates were applied to leaves which were exposed to 0–48 h intervals of moisture. They were also applied to leaves which remained from 0 to 36 h without wetness. All isolates efficiently colonized leaves, regardless of moisture interval or the delay to begin wetness. Although all isolates survived in green leaves of whole plants, colonization decreased throughout a 49-day period.

Conclusions: Brazilian isolates of *Cr* can establish and colonize strawberry leaves under a wide range of temperature and moisture conditions.

Significance and Impact of the Study: It is expected that the Brazilian isolates of *Cr* will establish efficiently in strawberry leaves where they can compete with *B. cinerea*.

Introduction

Botrytis cinerea Pers.: Fr. induces grey mould in many cultivated plants and causes severe losses, mainly in greenhouse crops (Jarvis 1992). Grey mould is difficult to control because *B. cinerea* can infect crops in almost all developmental stages, it has a wide host range, fungicide-resistant populations are common and epidemics progress with high infection rates (Sutton *et al.* 1997).

Biological control is considered as an effective component of grey mould management (Sutton *et al.* 1997; Morandi *et al.* 2003). *Clonostachys rosea* (Link: Fr.) Schroers, Samuels, Siefert & Gams (formerly *Gliocladium roseum* Bainier) (*Cr*) reduces *B. cinerea* growth and sporulation through hyperparasitism and competition for nutrients and through colonization of senescing/dead tissues (Sutton *et al.* 1997; Morandi *et al.* 2001). Successful biocontrol studies involving *Cr* were conducted, and isolate Pg 88-710 was selected in Canada as an efficient antagonist (Peng and Sutton 1991). This isolate suppressed *B. cinerea* sporulation and controlled grey mould in several hosts (Peng and Sutton 1991; Sutton *et al.* 1997, 2002; Morandi *et al.* 2003). Regardless of developmental stages of rose tissues, *Cr* established and suppressed *B. cinerea* sporulation (Morandi *et al.* 2000) even under conditions favourable for the pathogen (Morandi *et al.* 2003), while the ecological requirements for both pathogen and antagonist were similar (Morandi *et al.* 2006).

To be successful, a biocontrol agent must survive under different environmental conditions. Pg 88-710 survived 1 month in rose leaves under temperature ranging from 10 to 30°C (Morandi *et al.* 2001). To date, there is no clear demonstration of endophytic establishment of *Cr* in plant tissues, but there is strong evidence that related species can establish endophytically (Philipson 1991). Furthermore, *Cr* is considered to be able to colonize several

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host plants and different plant tissues without causing symptoms (Sutton *et al.* 1997). Thus, to increase antagonist survival, application of *Cr* on green leaves should be used to explore the potential endophytic establishment capacity (Sutton *et al.* 1997; Morandi *et al.* 2001). Although Cr can survive in leaves, experimental results on temporal dynamics of the antagonist on green tissues are scarce.

The success of *B. cinerea* biocontrol with Pg 88-710 motivated the search for isolates of *Cr* native to Brazil. Four isolates successfully established in rose, strawberry, eucalyptus, and tomato leaves, where they suppressed *B. cinerea* sporulation (Nobre *et al.* 2005). As in other reports (Peng and Sutton 1991; Kalogiannis *et al.* 2006), initial mass selection trials with Brazilian isolates were conducted under highly favourable conditions to *Cr*. Environmental conditions affect colonization, survival and activities of biocontrol agents (Sutton *et al.* 1997; Fravel 2005). Reduced field efficiency of biocontrol agents may be related to poor knowledge of their ecological requirements. Understanding antagonists' ecological requirements for establishment may help devise strategies for its application in agro ecosystems. Thus, we evaluated the effects of temperature and moisture in the colonization of strawberry leaves by the Brazilian isolates of *Cr* and their temporal dynamics in strawberry plants.

Materials and methods

General procedures

Strawberry plants (*Fragaria ananassa* 'Dover') were cultivated in a greenhouse in 20 cm-diameter plastic pots filled with soil : cow manure : sand (11 : 4 : 1 w/w), previously disinfested with methyl bromide and fertilized (5 g of 4-14-8 NPK pot^{-1}) every 60 days. Flower petals and old leaves were removed weekly to reduce chances of *B. cinerea* infection. Only fully expanded 20- to 30-day old green leaves were used.

In all experiments, we compared the four Brazilian isolates of *Cr* (NCR19/F, NCR60/F, NCR61/F and NCR62/F) selected by Nobre *et al.* (2005). Pg 88-710 was included in most experiments. Each isolate was grown in potato dextrose agar medium (PDA) at 25°C, under white light (15 μmol cm^{-2} s^{-1}), 12 h photoperiod. Conidia from 10 to 14-day old colonies were suspended in sterile distilled water (SDW) + Tween 20 (0·05% v/v), filtered through two layers of cheesecloth and adjusted to a concentration of 10^6 conidia ml^{-1}.

Conidial suspensions were sprayed on leaves or leaf discs with a DeVilbiss sprayer No. 15. All experiments included a control treatment in which leaves were sprayed with SDW + Tween 20. Data from this treatment (always zero values) were not included in statistical analysis.

Colonization of *Cr* was indirectly assessed by quantifying sporulation on leaf tissues (SLT). To quantify SLT, leaf discs (1 cm diameter) were cut and transferred, without any treatment, to paraquat–chloramphenicol–agar medium (PCA) (Peng and Sutton 1991) in Petri dishes. After 10–12 days incubation at 20°C, percentage of disc area with *Cr* sporulation was assessed under a stereoscope with a diagrammatic scale (Nobre *et al.* 2005).

Effect of temperature on mycelial growth *in vitro* and colonization of strawberry leaves by *C. rosea*

Mycelial plugs (5 mm diameter) from actively growing colonies of *Cr* in PDA were placed in PDA in Petri dishes, incubating at 10, 15, 20, 25, or 30°C. Colony radial growth was measured after 19 days when at least one colony had reached the dish borders. The experiment was run in a factorial design with five replicates (one dish = one experimental unit).

Conidial suspensions of each isolate were sprayed on groups of 15 leaf discs that were placed on PCA in Petri dishes and incubated at 10, 15, 20, 25 or 30°C. Disc colonization by *Cr* was quantified as described. The experiment was in a factorial design with four replicates (one dish = one experimental unit).

Effect of wetness duration on colonization of strawberry leaves by *C. rosea*

Two leaflets were placed in a transparent plastic box (11 cm width × 11 cm length × 3·5 cm depth – gerbox) on a 2-mm-nylon screen above a 1 cm-thick foam sponge soaked with SDW. Conidial suspensions were sprayed on to leaflets and incubated at 20°C, 12 h photoperiod. After 0, 6, 12, 24, 36 or 48 h of wetness duration, leaflets were air-dried for 30 min, discs were cut, and SLT was quantified. The experiment was in a factorial design with three replicates (a gerbox = one experimental unit).

Effect of postinoculation dry periods on colonization of strawberry leaves by *C. rosea*

Two leaflets were placed over dry foam in a gerbox. Spore suspension was sprayed on to leaflets and the gerboxes were left opened until start times of wetness (0, 2, 4, 8, 16, 24 or 36 h). To start this period of wetness, the foam was soaked with SDW and the gerboxes were closed and kept at 20°C, 12 h photoperiod. After 24 h of wetness, discs were cut and SLT was quantified. The experiment was in a factorial design with three replicates (a gerbox = one experimental unit).

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Colonization and survival of *C. rosea* in strawberry plants

Conidial suspensions were sprayed on fully expanded leaves still attached to plants; these were then transferred to a moist chamber (25°C, 12 h photoperiod) and after 24 h were returned back to the greenhouse. Two leaflets were detached per experimental unit (two plants) at 1, 7, 14, 21, 28, 36, 42 and 49 days after application, discs were cut, and SLT was quantified. The experiment had six treatments (five isolates plus the control) and four replicates.

Data analyses

All experiments were carried out twice, in a completely randomized design. Area under the *Cr* sporulation curve (AUCSC) for the experiment with whole plants was estimated (Morandi *et al.* 2003). After analysis of variance, comparisons of mean values were by the Protected Fisher LSD test ($\alpha = 0{\cdot}05$). Regression models were also adjusted, depending on their goodness of fit on determination coefficients (R^2), error mean squares, parameter significance, normality and unbiased distribution of residuals. Data were statistically analysed with SAS v. 9.1.

Results

Unless stated otherwise, variances of both experimental runs were similar according to Levene's test ($P > 0{\cdot}05$) and data were thus pooled. In all experiments, the interactions between isolates and treatments were not significant (all $P > 0{\cdot}20$). Thus, only the main effects were analysed.

Effect of temperature on mycelial growth *in vitro* and colonization of strawberry leaves by *C. rosea*

Mycelial growth was significantly different among isolates ($P = 0{\cdot}027$) (Table 1) and temperature levels ($P < 0{\cdot}0001$). A cubic equation best modelled the effect of temperature on growth (Fig. 1a). Maximum growth was estimated at 25·07°C.

Leaf colonization was not different among isolates (Table 1). Colonization was affected by temperature ($P < 0{\cdot}0001$), and a cubic model best fitted the data (Fig. 1b). Maximum colonization was estimated at 25·6°C.

Effect of wetness duration on colonization of strawberry leaves by *C. rosea*

Leaf colonization differed ($P = 0{\cdot}028$) among isolates (Table 1) and was not affected ($P = 0{\cdot}144$) by length of wetness period (Fig. 2a).

Effect of postinoculation dry periods on colonization of strawberry leaves by *C. rosea*

Leaf colonization differed among isolates ($P = 0{\cdot}025$) (Table 1) and time at which wetness began ($P = 0{\cdot}007$). SLT tended to increase as the wetness start interval increased (Fig. 2b), but the regression analysis was not significant, with low data fit ($R^2 = 0{\cdot}025$).

Colonization and survival of *Clonostachys rosea* in strawberry plants

For all isolates, SLT was greatest 1 day after application and decreased abruptly (50%) by 14 days after application. Less pronounced reduction occurred between 14 and 36 days. Lowest SLT occurred after 49 days post-application (Fig. 3). For AUCSC, variances of both runs were not equal. In neither run did isolates differ with respect to AUCSC values.

Discussion

Both '*in vitro*' mycelial growth and colonization of leaf tissues by all *Cr* isolates were affected by temperature,

Table 1 Comparison of isolates of *Clonostachys rosea* in four experiments that evaluated the effect of temperature on mycelial growth *in vitro*, as well as the effects of temperature, duration of wetness and postinoculation dry periods on colonization of strawberry leaves. The interactions between isolate and environmental factors were not significant in any of the experiments

	Mycelial growth (cm)	Colonization of strawberry leaves (%)		
Isolate	Temperature experiment	Temperature experiment	Wetness experiment	Dry periods experiment
NCR61/F	5·32a*	10·52a	10·81b	11·87ab
NCR19/F	5·18a	11·21a	12·29ab	11·40b
NCR60/F	5·11ab	11·70a	13·39a	12·86a
NCR62/F	4·89b	11·49a	11·75ab	12·79a
Pg 88-710	Not tested	10·74a	11·10b	11·81ab

*In each experiment, mean values with the same letter do not differ significantly (protected LSD, $\alpha = 0{\cdot}05$).

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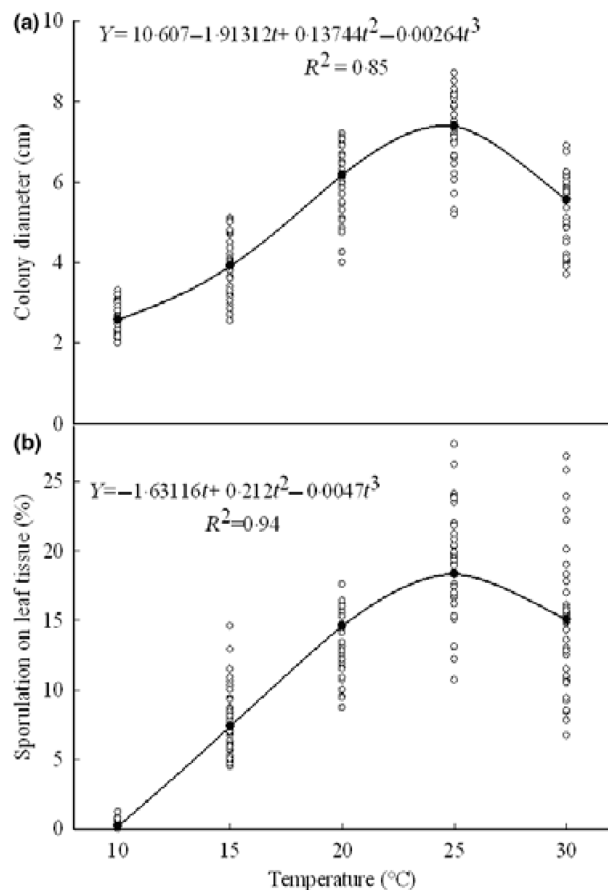

Figure 1 Mycelial growth (a) and sporulation on strawberry leaf discs (b) of *Clonostachys rosea* at different temperatures. (a) Colony diameter was measured after 19 days of growth in potato dextrose agar in Petri dishes. (b) Leaf discs were sprayed with conidial suspensions and immediately placed on paraquat–chloramphenicol–agar medium and incubated for 12 days. Intercepts and slopes of both models differed significantly from zero ($P < 0.05$). The symbols are: (○) observed and (●) estimated values.

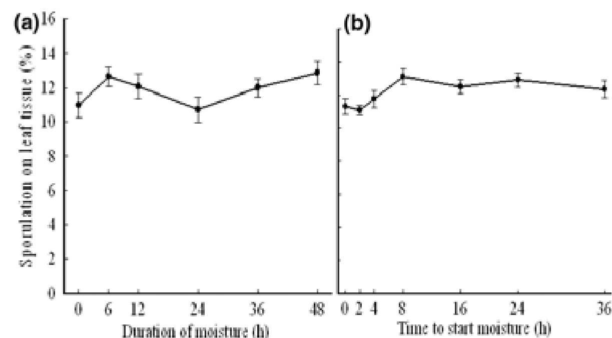

Figure 2 Sporulation of *Clonostachys rosea* on strawberry leaf discs that had been sprayed with conidial suspensions, kept at different durations of leaf wetness (a) or at different intervals to the start of leaf wetness (b), and incubated for 12 days at 25°C. Error bars represent standard errors of the mean values.

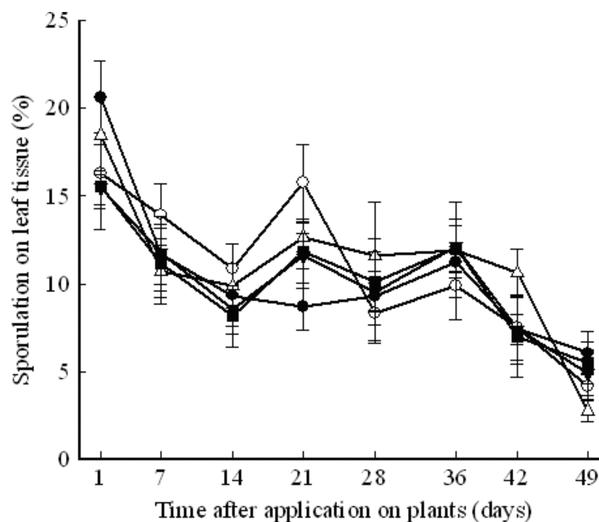

Figure 3 Sporulation of five *Clonostachys rosea* isolates on strawberry leaf discs. Plants were sprayed with conidial suspensions and leaves were sampled from 1 to 49 days after application. Error bars represent standard errors of the mean values. The symbols for the isolates are: (●) NCR19/F; (○) NCR60/F; (▼) NCR61/F; (△) NCR62/F and (■) Pg 88-710.

which was the most limiting factor to *Cr* development, as previously reported (Sutton and Peng 1993; Sutton *et al.* 1997; Yu and Sutton 1998; Köhl *et al.* 1999; Guetsky *et al.* 2001). Our results are similar to those obtained with raspberry leaves: Pg 88-710 sporulated poorly at 10–15°C even with 32 h of wetness duration (Yu and Sutton 1998). These findings affect strategies for the introduction of biocontrol agents. At low temperatures, more effective control methods such as fungicide applications alone or combined with biocontrol agents should be used (Buck 2004). Another possibility is using biocontrol agents that are efficient at low temperatures, such as *Ulocladium atrum* (Köhl *et al.* 1999). At 10°C *Cr* isolates scarcely sporulated or failed to do so in leaf discs, but grew on PDA. Sporulation and mycelial growth could have been affected differentially by temperature. Different enzymes may regulate mycelial growth and sporulation in leaf discs, which could account for differential responses to temperature (Köhl *et al.* 1999). It seems that temperature effects on *Cr* depends on the host, because at 10°C Pg 88-710 colonized and sporulated in rose leaves for up to 1 month (Morandi *et al.* 2001). This reinforces the need to evaluate antagonist isolates on different plant species (Nobre *et al.* 2005).

Wetness was not as limiting to *Cr* as was temperature. Even in prolonged periods of leaf wetness that favour other epiphytic micro-organisms, Pg 88-710 colonized rose leaves (Morandi *et al.* 2000). Although we did not quantify them, colonies of filamentous fungi, yeasts and

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bacteria were more abundant on discs of the control than on discs treated with *Cr*. Under longer periods of wetness, there was a trend towards large numbers of penicilliate-type conidiophores and no reduction in leaf disc area with sporulation. This is opposite to that reported for Pg 88-710 in rose leaves kept wet for 36–48 h, in which a large amount of verticilate-type conidiophores and leaf area with weak sporulation were observed (Morandi *et al.* 2001). This differential response may be due to differences in leaf tissue properties. Rose leaf tissue is richer in fibre than strawberry leaves and probably also more prone to be colonized by *Cr*. The fact that wetness does not affect colonization of Brazilian isolates of *Cr* in strawberry leaves may confer a competitive advantage upon them. Ideally, an antagonist should become established regardless of the duration of wetness periods. The antagonist must also be able to establish under prolonged wetness periods that favour infection and sporulation of *B. cinerea* (Blanco *et al.* 2006). The ability to establish in strawberry under short periods of wetness also promotes the success of a biocontrol agent under field conditions, in which antagonists face variations in relative humidity and leaf wetness (Fravel 2005). If inocula of both antagonist and pathogen are available, *Cr* requires a shorter duration of wetness and is likely to establish ahead of *B. cinerea*. Furthermore, all Brazilian isolates of *Cr* survived for 36 h in relatively low moisture environments such as in the growth chamber where relative humidity varied from 50% to 60%. This reinforces the low requirements of *Cr* regarding moisture. Similar results were found with Pg 88-710 on rose leaves (Morandi *et al.* 2001). However, Pg 88-710 sporulated poorly in raspberry leaves under wetness periods shorter than 8 h and temperatures between 10 and 30°C (Yu and Sutton 1998). In raspberry leaves, only endophytic growth of *Cr* was assessed and leaf discs were disinfested before transferring to PCA. As we did not disinfest leaf discs, probably the antagonist epiphytic growth was important for strawberry tissue colonization.

The ability of *Cr* to establish in leaves with no water film at high temperatures (25–30°C) is important for survival between or within growing seasons. These conditions favour *B. cinerea* in several hosts, including strawberry (Blanco *et al.* 2006). Survival of *Cr* in crop debris deserves attention. In rose leaves, Pg 88-710 survived for a month in temperatures from 10 to 30°C (Morandi *et al.* 2001). However, we found that in green strawberry leaves, colonization was reduced by at least 50% after 28 days. Temporal dynamics need to be re-evaluated in whole plants and crop debris to determine the ability of *Cr* to establish and survive. *Botrytis cinerea* can survive and sporulate in crop debris for up to 1 year (Araújo *et al.* 2005); thus, the longer the antagonist survives in such debris, the fewer sprays are required to control grey mould.

To be used successfully for the biocontrol of pathogens on aerial plant parts, antagonists must establish in the phylloplane. Brazilian isolates of *Cr* established in strawberry plants, although leaf colonization decreased with time. Apparently the decrease in leaf colonization follows a pattern common to other biocontrol agents (Köhl *et al.* 2000; Guetsky *et al.* 2002; Freeman *et al.* 2004). *Clonostachys rosea* seems to be able to establish either endophytically or epiphytically on plant organs (Sutton *et al.* 1997; Nobre *et al.* 2005). In our experiments, *Cr* growing as an epiphyte on strawberry leaves probably survived less than as an endophyte, a condition in which it was more protected. Increases in colonization efficiency could be obtained by appropriate formulations, as *Cr* conidia need external sources of nutrients to germinate (Morandi *et al.* 2001).

Brazilian isolates of *Cr* were just as effective as Pg 88-710 in colonizing strawberry leaves. These isolates are expected to be efficient in competing with *B. cinerea* in strawberry as well as in other hosts. We are evaluating the ability of Brazilian isolates of *Cr* in reducing *B. cinerea* under cultivation conditions.

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