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Effects of host and microbial factors on development of *Clonostachys rosea* and control of *Botrytis cinerea* in rose

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Abstract

Development of *Clonostachys rosea* in rose leaves and petals and control of *Botrytis cinerea* by the agent were investigated. *C. rosea* germinated, established endophytic growth, and sporulated abundantly whether the tissues were mature, senescent or dead when inoculated. Germination incidence was moderate on mature and senescent leaves (47% and 35%) and petals (31% and 43%), and high (> 98%) on dead tissues. Sporulation of *C. rosea* in tissues inoculated when mature, senescent or dead averaged 41%, 61%, and 75% in leaves, and 48%, 87% and 53% in petals. When leaves were wounded with needles before inoculation, germination of *C. rosea* increased from 45–56% to 90–92%, but sporulation became high (> 75%) regardless of wounds. When leaves were inoculated with *C. rosea* at 0–24 h after wounding and subsequently with *B. cinerea*, germination of the pathogen was reduced by 25–41% and sporulation by ≥ 99%. A humid period prior to inoculation of senescent or dead leaves promoted communities of indigenous fungi, reduced sporulation of *C. rosea* and *B. cinerea*, and, in dead leaves, increased control of the pathogen associated with *C. rosea*. Applied at high density, isolates of indigenous *Penicillium* sp. and *Alternaria alternata* from rose interacted with *C. rosea* and reduced control of the pathogen by 16% and 21%, respectively. In conclusion, *C. rosea* markedly suppressed sporulation of *B. cinerea* in rose leaves and petals regardless of developmental stage, minor wounds, and natural densities of microflora. This versatility should allow *C. rosea* to effectively control inoculum production of *B. cinerea* in rose production systems.

Introduction

Botrytis cinerea Pers.: Fr. is a destructive pathogen of hybrid roses (*Rosa hybrida* L.) grown as cut flowers in greenhouses in Canada, Brazil, and other countries (Horst, 1983; Elad, 1988; Hammer and Evensen, 1994; Tatagiba et al., 1998). Flecks, spots, and blighting produced by *B. cinerea* in the petals are important factors contributing to economic losses in roses. However, the pathogen also causes stem cankers and can produce lesions on the leaves and vegetative buds (Horst, 1983). Sanitation, cultural practices, microclimate regulation, fungicide sprays and treatment of the flowers with antioxidants, growth regulators, or warm water are measures used against *B. cinerea* in rose production systems (Horst, 1983; Elad, 1988; Hammer and Marois, 1989; Elad and Volpin, 1991; Elad et al., 1993; Elad and Evensen, 1995; Hausbeck and Moorman, 1996). Some rose cultivars are moderately resistant to the pathogen (Hammer and Evensen, 1994). Nonetheless, practical control of *B. cinerea* in roses when in the greenhouse or in postharvest storage or transit often fails to meet industry requirements. Chemical fungicides, though widely employed, are in many instances under use restrictions or have declined in effectiveness because of increased insensitivity of the pathogen (Jarvis, 1992; Hausbeck and Moorman, 1996). In attempts to improve disease control, several

investigators have explored use of microbial agents (Redmond et al., 1987; Hammer and Marois, 1989; Elad et al., 1993; Tatagiba et al., 1998).

One of the agents with superior performance against *B. cinerea* is a fungus long known as *Gliocladium roseum* Bainier (Sutton et al., 1997), but which was reclassified recently as *Clonostachys rosea* (Link: Fr.) Schroers, Samuels, Siefert and W. Gams [teleomorph, *Bionectria ochroleuca* (Schw.) Schroers and Samuels] (Schroers et al., 1999). In a recent study in Brazil, *C. rosea* completely blocked sporulation of the pathogen in rose leaf residues and was the most effective among 52 diverse microbes evaluated (Tatagiba et al., 1998). *C. rosea* was among a few microbes that suppressed the pathogen by more than 90% in detached rose petals. None of the microbes investigated, or those reported in other studies (Redmond et al., 1987; Elad et al., 1993), suppressed symptoms in rose flowers by more than 50–65%, so are unlikely to be commercially acceptable for direct protection of the flowers. However, because *C. rosea* strongly suppresses inoculum production of *B. cinerea* in residues of rose and other crops, it might be feasible to employ the agent to protect rose flowers by reducing inoculum density of the pathogen.

The chief inoculum sources of *B. cinerea* in greenhouses are dead foliage and petals of rose which accumulate on the soil or greenhouse floor. In Brazil, the pathogen was able to sporulate for at least 8 months in leaf residues and for a year in petal residues placed on the surface of rose beds in commercial greenhouses (E. de A. Araújo, 1995, personal communication). An important strategy to reduce sporulation potential of *B. cinerea* in residues is to control infection and colonization of the foliage and flowers by the pathogen. Besides infecting nonwounded foliage and flowers, *B. cinerea* often infects tissues wounded by pruning, flower harvesting, and other production practices (Horst, 1983; Jarvis, 1992; M.A.B. Morandi, unpublished observations). Infections arising from conidia in most instances are initially quiescent, but become aggressive when the tissues senesce or are stressed. In contrast, infections initiated by mycelium in plant residues, such as dead petals adhering to healthy foliage, become aggressive immediately (Elad, 1988; Jarvis,1992; Williamson, 1994). Sporulation of *B. cinerea* on colonized rose residues requires favorable temperature and humid periods (E. de A. Araújo, personal communication).

A nonpathogenic endophyte, *C. rosea* is able to suppress development and sporulation potential of *B. cinerea* in plant tissues through mechanisms such as hyperparasitism, nutrient competition and competitive colonization of senescing or dead tissues (Sutton et al., 1997; Yu and Sutton, 1997; Köhl and Fokkema, 1998). Effectiveness of the agent against the pathogen, however, is known to be influenced by host factors and host-associated microflora (Sutton, 1995; Sutton et al., 1997; Yu and Sutton, 1997). An understanding of the host and indigenous microflora in relation to the development of *C. rosea* and its ability to suppress *B. cinerea* is needed for development of rational programs to optimize the timing and targetting of *C. rosea* treatments. Accordingly, studies were conducted to determine the effects of the developmental stage of leaves and petals, tissue wounds, and host-associated microflora on germination, growth, and sporulation of *C. rosea* on the host and the ability of the agent to reduce inoculum production by *B. cinerea* in leaf and petal residues.

Materials and methods

Rose plants

Plants of rose, cv. Sonia, were produced in 20-cm-diameter pots containing a soilless mix (Promix®, Plant Products Ltd., Brampton, Ontario, Canada) in a climate-controlled greenhouse. Average air temperatures in the greenhouse were 19–25 °C during the day between 0800 and 2000 h and 16–21 °C during the night between 2000 and 0800 h. White shades in the greenhouse roof were opened when irradiance was less than 800 $\mu mol\, m^{-2}\, s^{-1}$ and closed when it exceeded 1200 $\mu mol\, m^{-2}\, s^{-1}$, and at night to reduce heat loss. Plants were supplied with soluble N : P : K (20 : 8: 20) fertilizer (150 $g\, l^{-1}$ of water) once a week.

Inoculum production and inoculations

Isolate PG-88-710 of *C. rosea* (Peng and Sutton, 1991) and isolate MM-98-1 of *B. cinerea* from a flower of a greenhouse-grown rose were used for inoculations. Conidia of each fungus were produced on potato dextrose agar (PDA) medium under cool-white fluorescent lamps (12-h photoperiod) at 20–22 °C. The conidia were suspended in sterile distilled water plus surfactant (0.05 ml Triton X-100/100 ml), filtered through three layers of cheesecloth and diluted to a desired density. For inoculations, the inoculum density of *C. rosea* was 10^7 conidia ml^{-1} and that of *B. cinerea* was 10^6 conidia ml^{-1}. Inoculum was agitated continuously before

application, and was applied as 10 μl droplets to 1-cm-diameter leaf or petal disks or entire leaflets or petals by means of an automatic pipette.

Germination and germ tube growth

To estimate germination incidence and germ tube length of *C. rosea* and of *B. cinerea*, 1-cm-diameter disks of inoculated leaves or petals were mounted in lactophenol containing 0.05% trypan blue on microscope slides, gently heated over a flame for 2 min to clear the tissues, and examined on a compound microscope (Saha et al., 1988). Germination incidence was estimated from observation of 100 conidia on each disk. A spore was considered germinated when length of the germ tube exceeded the greatest diameter of the spore. Germ tube length was measured in 10 germinated conidia selected arbitrarily on each disk, except when tubes were long, intermingled, and not easily distinguishable.

Endophytic development

Endophytic establishment or colonization by *C. rosea* and *B. cinerea* in the host were evaluated indirectly by quantifying the potential of the fungi to sporulate on the tissues. To estimate sporulation potential, tissues were disinfested in 70% ethanol for 10 s and in 2.0% sodium hypochlorite (5.95% Javex) for 10 s, rinsed three times in sterile distilled water, and transferred to paraquat-chloramphenicol agar (PCA) medium in Petri dishes (Peng and Sutton, 1991). Sporulation was estimated after the tissues has been incubated at 25 °C for 4, 7, and 11 days.

Sporulation

Sporulation of *C. rosea* and *B. cinerea* on host tissues was quantified by estimating the percent tissue area with conidiophores of the respective fungi. To estimate sporulation of *C. rosea*, an eight-category scale developed previously from direct counts of conidiophores on 1-cm-diameter disks of rose leaves was used (M.A.B. Morandi and L.A. Maffia, unpublished). The categories with equivalent mean percent area of tissues with conidiophores and, in parentheses, the range of percent tissue area with conidiophores and mean number of conidiophores per disk were as follows: 0 = 0% (0%, 0); 1 = 2% (1–3%, 7.5); 2 = 5% (4–6%, 18.7); 3 = 10% (7–13%, 37.5); 4 = 20% (14–27%, 75.0); 5 = 40% (28–52%, 150); 6 = 70% (53–87%, 263); and 7 = 94% (88–100%, 353). To estimate sporulation of *B. cinerea*, an eight-category scale was used (Peng and Sutton, 1991). The categories with equivalent mean percent area of tissues with conidiophores and, in parentheses, the range of percent tissue area with conidiophores and mean number of conidiophores per disk were as follows: 0 = 0% (0%, 0); 1 = 2% (1–3%, 6.5); 2 = 5% (4–6%, 18.5); 3 = 10% (7–12%, 36.5); 4 = 20% (13–26%, 74.5); 5 = 40% (27–53%, 150.5); 6 = 65% (54–76%, 250.5), 7 = 90% (77–100%, 350.5). For each scale, mean percent values were used for data analysis.

Developmental stages of leaves and petals

Leaves and petals used for various experiments were categorized as mature, senescent, and dead. Mature leaves were green, fully expanded, and attached to the host plant when selected for use. Senescent leaves were yellow and attached. Mature petals were from outer portions of harvest-ready flower buds, and senescent petals were taken from flowers that had been fully opened for 4–5 days. Dead leaves and petals were dry, pale brown, unfragmented, lacked sporulation of microfungi, and had fallen 4–6 days prior to collection.

Effects of developmental stage of host organs

Growth and sporulation of *C. rosea* was investigated in leaves and petals at each of the three developmental stages. Groups of 1-cm-diameter disks of leaf laminae, 1-cm-long segments of leaf petioles, and 1-cm-diameter petal disks were prepared. Disks or segments of each group were placed on fiberglass screen (1 mm mesh) over moist paper towels in Petri dishes. A 10 μl droplet of *C. rosea* inoculum, or of water plus surfactant only, was placed near the center of each disk or segment, and the tissues were incubated at 25 °C. There were 60 leaf disks, 18 petiole segments, and 60 petal disks of each developmental stage arranged in a completely randomized design. Germination incidence, germ tube growth, and endophytic development of *C. rosea* were estimated in the various tissues. Germination and germ tube growth were quantified on five leaf disks and five petal disks taken from within each group at 14 and 24 h after inoculation. To estimate endophytic development, arbitrarily selected leaf disks, petal disks and petiole segments of each group were surface disinfested and placed on PCA at 48 h

after inoculation. Percent area of tissues with conidiophores of *C. rosea* was estimated for 20 leaf disks, 20 petal disks, and 12 petiole segments after 4, 7, and 11 days of incubation.

Effects of wounds

Tissue wounds were investigated in relation to growth and sporulation of *C. rosea* in mature leaves that were detached and washed twice or not washed. For washing, each leaf was shaken in 250 ml sterile water for 1 min and air dried. Ten leaflets from the washed leaves and 10 from unwashed leaves were wounded by means of a needle apparatus. The apparatus comprised stainless-steel needles 32 mm long and 0.2 mm diameter mounted in a plastic holder. Eight needles were mounted with their points spaced equidistantly in a circle of 5 mm diameter, and one needle was at the center of the circle. For each leaflet the apparatus was used to make nine holes, each about 0.2–0.3 mm diameter, at each of five sites that were approximately equidistant from each other and from the leaflet margin. Ten other leaflets from washed leaves and 10 from unwashed leaves were not wounded. Leaflets were inoculated by placing a 10 µl droplet of *C. rosea* inoculum on each wound site and at equivalent sites in nonwounded leaflets, and immediately placed in high humidity in Petri dishes. Five leaflets of each treatment were removed from the humid chambers after 14 h and five after 24 h. At each time of sampling, one disk was cut at an arbitrary inoculation site on each leaflet and used for estimating germination and germ tube growth of *C. rosea*. Two other disks from each leaflet were used to estimate endophytic development.

In a similar study, wounds and wound age were investigated in relation to development of *C. rosea* and *B. cinerea* inoculated alone and in combination in unwashed leaflets of mature leaves. Leaflets were either not wounded, or were wounded at 0, 1, 3, 6, and 24 h before inoculation. For inoculation, droplets of *C. rosea* or *B. cinerea* inoculum were positioned over wounds or on equivalent sites on nonwounded leaflets. Leaflets inoculated with the agent or pathogen only were kept at high humidity in Petri dishes for 24 h after which germination, germ tube growth, and endophytic development were estimated for each fungus. For combined inoculations, leaflets were inoculated with *C. rosea*, kept in high humidity for 24 h, air dried, challenge-inoculated with *B. cinerea*, and kept in high humidity for a further 24 h. Germination, germ tube length (*B. cinerea* only) and endophytic development were then estimated in disks taken from five leaflets per treatment.

Effects of a preinoculation humid period

Senescent and dead leaflets were inoculated with *C. rosea* or with *B. cinerea*, or were treated only with water plus surfactant, immediately or after 24 h in high humidity at 25 °C. After inoculation, leaflets were kept at high humidity at 25 °C for 24 h after which surface moisture was air dried. Some leaflets that were treated with *C. rosea*, incubated in high humidity, and air dried for 30 min, were immediately challenge-inoculated with *B. cinerea* and incubated and air dried as before. Five 1-cm-diameter disks were cut from each of five leaflets in each treatment. One disk from each leaflet (five disks per treatment) was used to estimate germination and germ tube growth of *C. rosea* and *B. cinerea*, and the remaining four disks (20 disks per treatment) were transferred to the PCA medium for estimation of tissue areas with conidiophores of *C. rosea*, *B. cinerea*, and indigenous fungi. Tissue area with sporulation of indigenous fungi was estimated by means of an equi-incremental scale (0%, 1–10%, . . . , 91–100%).

Effects of indigenous microfungi

One isolate of each of three chief fungi found on the leaves was examined in relation to development of *C. rosea* and *B. cinerea* on the host. Leaflets detached from green leaves were inoculated with *Penicillium* sp. or with *Aspergillus* sp. (each at 10^7 conidia ml^{-1} water plus surfactant), or with *Alternaria alternata* (5×10^5 conidia ml^{-1}), incubated in high humidity at 25 °C for 24 h and air dried. Leaflets treated with each fungus were inoculated with *C. rosea*, *B. cinerea*, *C. rosea* plus *B. cinerea*, or with water plus surfactant, incubated in high humidity, and assessed for germination, growth, and sporulation.

Experimental design and data analysis

Each experiment was conducted with a completely randomized design and repeated once. Statistical computations were performed using the Statistical Analysis Systems (SAS Institute Inc., Cary, NC). Data for

conidial germination, length of germ tubes and fungal sporulation were examined using analysis of variance (ANOVA) and treatment means were compared by the protected least significant difference test (PLSD; Snedecor and Cochran, 1989). Analysis of data of the two experimental repetitions invariably resulted in treatment effects in the same significance classes. Accordingly, in all experiments, data of one repetition are presented.

Results

Effects of developmental stage of host organs

Germination incidence of *C. rosea* 14 h after inoculation was moderate on mature and senescent leaves (47% and 35%, respectively) and on mature and senescent petals (31% and 43%, respectively), but exceeded 98% on dead leaves and petals. More spores germinated on mature leaves than on senescent leaves, but germination on mature and senescent petals did not differ significantly. Germination incidence increased on mature and senescent leaves and petals when the humid period was extended to 24 h (Figure 1). At that time, germination on senescent leaves (93%) exceeded that on mature leaves (68%), while germination on mature and senescent petals was similar (60–61%) and well below that on dead petals (95%).

Germ tube length of *C. rosea* 14 h after inoculation was similar on dead leaves and petals (62–77 μm) and in each instance was greater than on mature and senescent tissues. At 24 h after inoculation, germ tubes were 240–250 μm on senescent and dead leaves and on dead petals, but shorter (52–58 μm) on mature leaves and on mature and senescent petals (Figure 1).

Sporulation of *C. rosea* was observed on laminae and petioles of leaves, and on petals, that were mature, senescent, or dead when inoculated. Observations for leaves transferred to PCA medium after 14 and 24 h of high humidity did not differ significantly, so were combined (Figure 2). Estimated mean area of leaf laminae with sporulation after 7 days on PCA was highest (75%) for dead leaves, intermediate (60%) for senescent leaves, and least (41%) for mature leaves. In petioles, tissue area with sporulation was high (73–90%) for mature, senescent, and dead leaves, and significantly higher for mature tissues than dead tissues. In petals, the sporulation area was high (87%) for senescent tissues, and moderate (48–53%) for mature and dead tissues; this pattern was found also after 11 days of incubation. The sporulation area on laminae and petioles of mature, senescent and dead leaves did not differ significantly after 11 days on PCA.

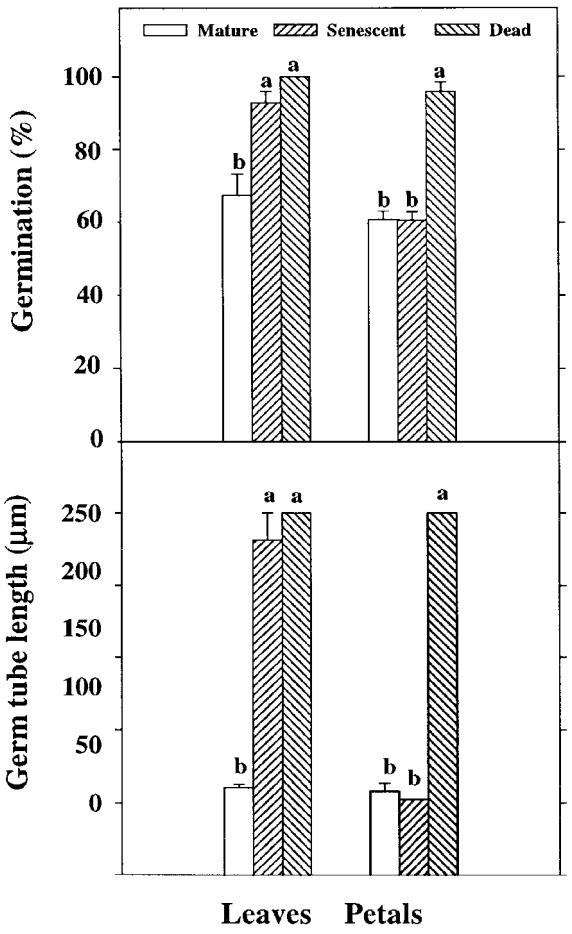

Figure 1. Estimated germination incidence and germ tube length of *Clonostachys rosea* in mature, senescent, and dead leaves and petals of rose at 24 h after inoculation. Data bars are mean values each with a standard error bar. Treatment means for a given host organ, followed by the same letter, are not significantly different (protected LSD, $P \leq 0.05$).

Effects of wounds

Germination, germ tube growth, and sporulation of *C. rosea* on leaves that were washed or not washed did not differ significantly ($P \leq 0.01$) so data for the respective variables were combined. Germination incidence (± SE) of *C. rosea* was much higher on wounded leaves than on nonwounded leaves at 14 h after inoculation (respectively 90 ± 2.7% and 45 ± 4.1%) and

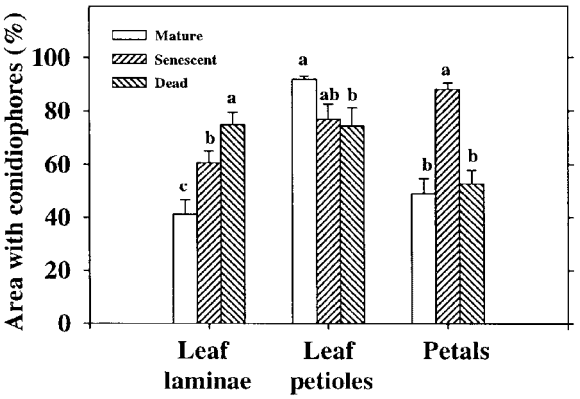

Figure 2. Estimated conidiophore production by *Clonostachys rosea* in leaf disks, petiole segments, and petal disks that were cut from mature, senescent, or dead rose tissues, inoculated with *C. rosea*, kept in high humidity, and incubated on an agar medium containing paraquat for 7 days. Data bars are mean values each with a standard error bar. Treatment means for a given host organ, followed by the same letter, are not significantly different (protected LSD, $P \leq 0.05$).

at 24 h after inoculation (respectively 92 ± 1.9%, and 56 ± 5.6%). At 14 h, germ tubes on wounded leaves were, on average, more than six times longer (158 ± 15 μm) than on nonwounded leaves (22 ± 2.6 μm), and at 24 h were more than four times longer (240 ± 6.2 μm compared to 55 ± 14.4 μm).

Wounding of leaves before inoculation with *C. rosea* significantly increased ($P \leq 0.05$) conidiophore production of the agent after the leaves were kept in high humidity for 14 h and transferred to PCA. As the period on PCA was lengthened from 4 to 11 days, the estimated area of tissues with conidiophores increased from 21% to 89% on wounded leaves, and from 7% to 75% on nonwounded leaves. Observations for leaves kept in high humidity for 24 h after inoculation were not significantly different ($P \leq 0.05$) from those removed at 14 h.

Almost all conidia of *C. rosea* (> 88%, data not presented) and of *B. cinerea* (92–94%) germinated on leaves inoculated with the respective fungi at 0, 1, 3, 6, or 24 h after wounding (Figure 3). When leaves inoculated with *C. rosea* were challenge-inoculated after 24 h with *B. cinerea*, germination incidence of the pathogen was high (91%) on leaves treated with *C. rosea* immediately after wounding, declined to 68% on those treated at 1 h after wounding, and stabilized at 53–55% on those treated at 3–24 h after wounding. Germ tubes of *B. cinerea* averaged 130–190 μm in length on wounded

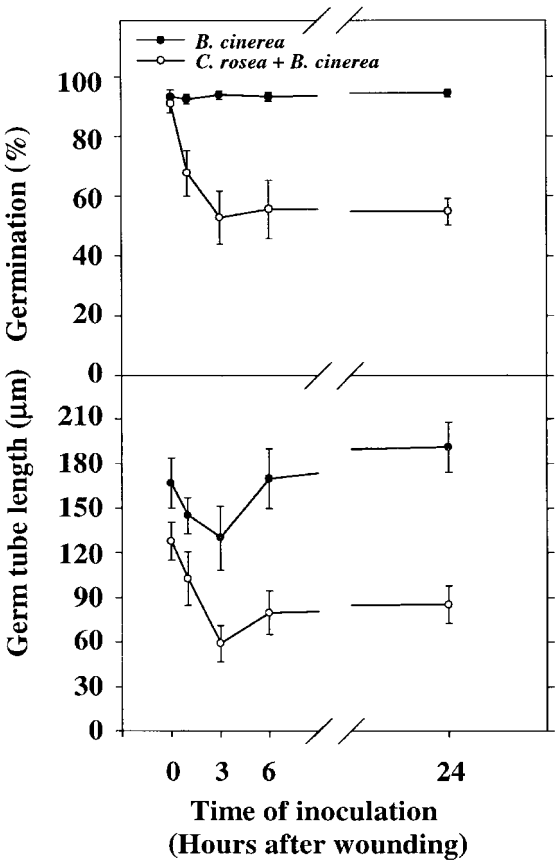

Figure 3. Estimated incidence of germination and length of germ tubes of *Botrytis cinerea* in disks of mature rose leaves that were wounded with a needle apparatus, and inoculated after various times with the pathogen, or with *Clonostachys rosea* followed after 24 h with *B. cinerea*. Curves show mean values with standard error bars.

leaves inoculated with the pathogen only. Germ tubes on leaves inoculated at 0–6 h after wounding were of similar length, but those on leaves inoculated after 24 h were longer (190 μm) than those inoculated at 1 or 3 h. Treatment of leaves with *C. rosea* before inoculation with *B. cinerea* reduced germ tube growth of the pathogen (Figure 3). Germ tubes of *B. cinerea* were 24% and 29% shorter, respectively, when *C. rosea* was applied to leaves at 0 and 1 h after wounding, and 50–58% shorter when applied at 3, 6 or 24 h, compared to leaves without *C. rosea*.

The time of inoculation of leaves following wounding with the needle apparatus had only minor effects on sporulation of *C. rosea* and *B. cinerea* after the tissues were incubated on PCA. Conidiophore production

by *C. rosea* when applied alone at 0–6 h after wounding was 73–84% and did not differ significantly ($P \leq 0.05$), but significantly less (67%) when applied at 24 h. Similarly, leaf areas with conidiophores of *B. cinerea* when the pathogen was applied alone at 0–6 h after wounding were 70–80% and not significantly different ($P \leq 0.05$), but lower (61%) when inoculated at 24 h. In the combined treatments, in which wounded leaves were inoculated with *C. rosea* and 24 h later with *B. cinerea*, the agent sporulated on 65–67% of leaf area when applied at 0 or 1 h after wounding and on significantly greater areas (77–80%) when applied at 3–24 h. Leaf area with sporulation of the pathogen was only 0–1%, regardless of the time of inoculation, when wounded leaves were treated with *C. rosea*.

Effects of a preinoculation humid period

A 24-h humid period prior to inoculation with *C. rosea* or with *B. cinerea* did not significantly affect germination of either fungus on the leaves ($P \leq 0.05$). Germination of *C. rosea* conidia was 70–80% on senescent leaves and 97–98% on dead leaves; respective values for *B. cinerea* were 63–70% and 95–99%. Application of *C. rosea* before *B. cinerea* significantly reduced germination of the pathogen by 18% and 33% ($P \leq 0.05$), respectively, on senescent leaves that did or did not receive a preinoculation humid period. On dead leaves, the agent significantly reduced germination of *B. cinerea* by 88% following a preinoculation humid period and by 62% in the absence of the humid period.

The preinoculation humid period also did not significantly affect ($P \leq 0.05$) lengths of germ tubes of *B. cinerea*, which averaged 33–54 μm on senescent leaves and 232–246 μm on dead leaves. Application of *C. rosea* prior to *B. cinerea* reduced germ tube length of the pathogen by 58–65% in senescent leaves and by 81–91% in dead leaves.

The preinoculation humid period did significantly reduce ($P \leq 0.05$) conidiophore production of *C. rosea* and *B. cinerea* on the leaves. In leaves inoculated with *C. rosea* only, preinoculation high humidity reduced estimated mean areas of disks with conidiophores of the agent after 11 days on PCA from 78% to 67% for senescent leaves and from 88% to 76% for dead leaves. In leaves inoculated with *B. cinerea* only, the humid period reduced conidiophores of the pathogen from 55% to 43% in senescent leaves, and from 69% to 51% in dead leaves. In senescent and dead leaves inoculated with *C. rosea* and challenged after 24 h with *B. cinerea*, estimated areas with conidiophores of the pathogen were extremely low (0.1–2.1% and 1.3–2.3% respectively), and not significantly affected by preinoculation high humidity ($P \leq 0.05$).

Incubation of leaves in high humidity for 24 h before tissues were transferred to PCA increased conidiophore production by indigenous fungi on the disks (Figure 4). Increases for dead leaves were greater than those for senescent leaves. Principal fungi sporulating on the tissues included species of *Penicillium, Aspergillus* and *Alternaria*.

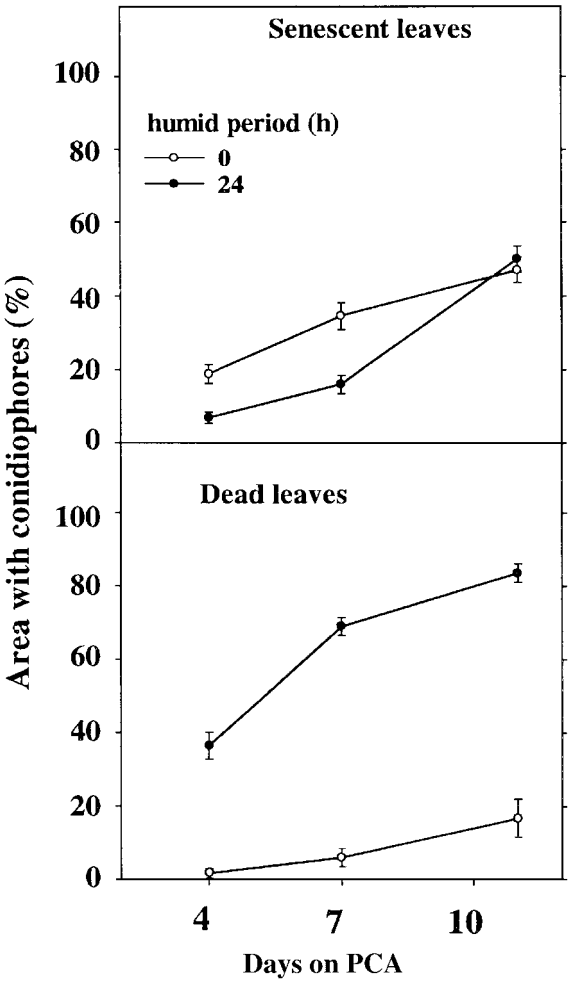

Figure 4. Effects of exposing senescent and dead leaves to high humidity for 24 h on conidiophore production by indigenous fungi in disks taken from the leaves and incubated for various periods on an agar medium containing paraquat (PCA). Curves show mean values plus standard error bars.

Effects of indigenous microfungi

Isolates of *Aspergillus* sp. and *Alternaria alternata* Fr.: Keissler, applied to green leaves 24 h before *B. cinerea*, each suppressed germination of *B. cinerea* markedly and as effectively as *C. rosea* (Figure 5). The isolate of *Penicillium* sp. was moderately suppressive. Combination treatment of *C. rosea* with *Aspergillus* sp. reduced germination of the pathogen to about the same level as did *Aspergillus* sp. alone. However, *C. rosea* increased suppression of *B. cinerea* by *Penicillium* sp. and dramatically decreased suppressiveness of *A. alternata*. Observations of the indigenous microfungi and *C. rosea* on germ tube growth of *B. cinerea* were highly variable and in most instances did not differ significantly.

Applied alone, *C. rosea* produced conidiophores on about 75% of leaf surface area (Figure 6). Sporulation of the agent was not affected significantly on leaves also inoculated with *B. cinerea*, *Aspergillus* sp., or *Penicillium* sp., but was greatly reduced by *A. alternata*.

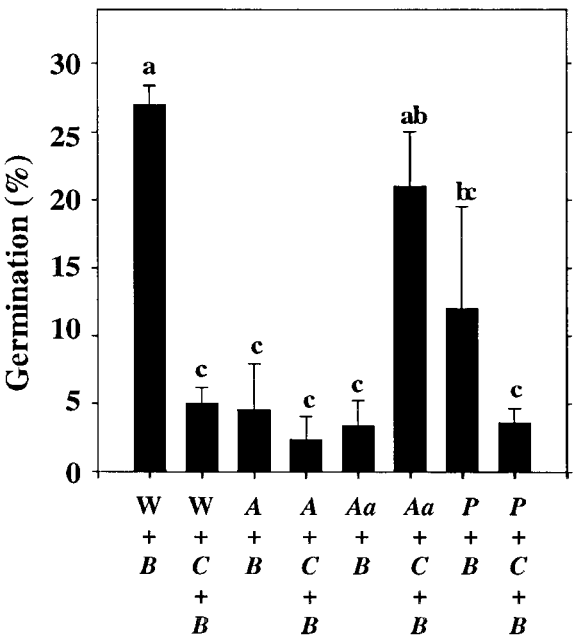

Figure 5. Germination incidence of *Botrytis cinerea* in green rose leaves that were treated with water plus surfactant (W), *Aspergillus* sp. (A), *Alternaria alternata* (Aa), or *Penicillium* sp. (P), alone or, after 24 h, also with *Clonostachys rosea* (C), and challenge-inoculated with the pathogen (B). Data bars are mean values each with a standard error bar. Data bars assigned the same letter are not significantly different ($P \leq 0.05$, protected LSD).

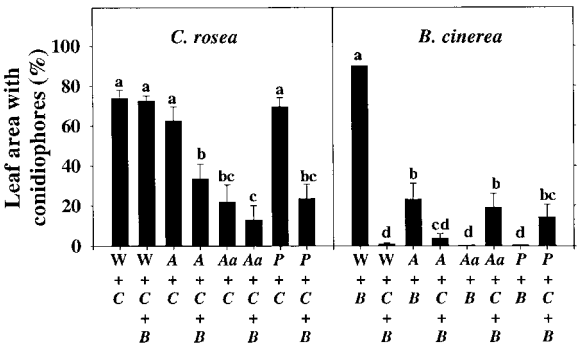

Figure 6. Effects of applications to green rose leaves of *Aspergillus* sp. (A), *Alternaria alternata* (Aa), *Penicillium* sp. (P) or water plus surfactant (W), plus after 24 h *Botrytis cinerea* (B), or *Clonostachys rosea* (C), or *C. rosea* plus after a further 24 h *B. cinerea*, on conidiophore production by *C. rosea* and *B. cinerea* in disks taken from the leaves and incubated on an agar medium containing paraquat. Data bars are mean values each with a standard error bar. Data bars assigned the same letter are not significantly different ($P \leq 0.05$, protected LSD).

Combinations of *Aspergillus* sp. with *B. cinerea* and *Penicillium* sp. with *B. cinerea* reduced sporulation of *C. rosea* compared to either fungus alone, but *A. alternata* with *B. cinerea* was not more suppressive than *A. alternata* alone.

Applied alone, *B. cinerea* produced conidiophores on 90% of inoculated leaf area, but on only 22% in leaves previously treated with *Aspergillus* sp. only, and on less than 1% in those previously treated with *A. alternata*, *Penicillium* sp., or *C. rosea* only (Figure 6). A combination of *Aspergillus* sp. with *C. rosea* was more suppressive than *Aspergillus* sp. alone, and as suppressive as *C. rosea* alone. However, combinations of *A. alternata* with *C. rosea* and of *Penicillium* sp. with *C. rosea* were, in each instance, less suppressive than either fungus alone. After leaves inoculated with combinations of *C. rosea* with *B. cinerea*, *Penicillium* sp., *Aspergillus* sp. or *A. alternata* and incubated on PCA for 20–25 days, abundant hyphae and conidiophores of *C. rosea* were present on colonies of the pathogen and each indigenous fungus, hyphae of which appeared deteriorated.

Discussion

Clonostachys rosea germinated, established endophytic growth, and sporulated abundantly in rose leaves and petals, whether the tissues were mature, senescent, or dead when inoculated. Germination and germ tube

growth were moderate or high on all tissues tested, though usually greater on dead tissues, possibly on account of greater nutrient availability. By inference from estimates of conidiophore production on tissues kept on PCA, colonization and sporulation potential of *C. rosea* were moderate to high for all leaf and petal tissues investigated. Previous evidence indicated that colonization of leaves and petals of various hosts by *C. rosea* is rapid and intensive chiefly when the tissues senesce and die, so that sporulation potential increases markedly at these stages (Sutton et al., 1997). Availability of nutrients in tissues likely influenced colonization density, and as a consequence, observed levels of conidiophore production. Findings that *C. rosea* sporulated heavily on leaves and petals inoculated at various developmental stages underscored a potential for sustained association of the agent with rose crops.

Wounding of rose leaves with the needle apparatus prior to inoculation vastly increased germination incidence and germ tube growth of *C. rosea* and accelerated conidiophore production by the agent when the tissues were incubated on PCA. Almost all conidia germinated on wounded leaves, whether applied immediately or at 1–24 h after wounding, whereas only about half germinated on nonwounded leaves. The effects of preinoculation wounding on *C. rosea* probably resulted in part from stimulation of the agent by nutrients leaking from wounded cells into wetness films on the phylloplane. In the absence of wounds, low nutrient availability may have limited germination and growth of *C. rosea* on leaves as was implicated in other hosts (Sutton et al., 1997; Yu and Sutton, 1997). In unpublished studies (M.A.B. Morandi, 1999), conidia of the agent germinated poorly or not at all in water on microscope slides, so may be dependent on exogenous nutrients. While conidiophores on nonwounded leaves developed about 24–36 h after those on wounded leaves, areas with conidiophores subsequently were similar regardless of wounding.

Competition for available nutrients probably contributed to the observed interactions between *C. rosea* and *B. cinerea* on the leaves. As in *C. rosea*, exogenous nutrients stimulate germination and growth of *B. cinerea* on host plants (Blakeman, 1980). Decreasing nutrient concentration in moisture films on wounded leaves with time after wounding, perhaps through increased dilution or utilization by indigenous microbes, may have intensified nutrient competition between *C. rosea* and *B. cinerea*. Greater nutrient competition might account for the observations that *C. rosea* was increasingly effective against a challenge inoculation of *B. cinerea* when the agent was applied to leaves at 1 and 3 h after wounding compared to 0 h. While *C. rosea* suppressed *B. cinerea* on the phylloplane, in some instances by 40–50% or more, the agent reduced conidiophore production of the pathogen by 99–100%, regardless of time between wounding and inoculations. Accordingly, antagonism during tissue colonization probably was of overwhelming importance for reducing sporulation potential of *B. cinerea*, as was concluded also in raspberry (Yu and Sutton, 1997).

Circumstantial evidence indicated that indigenous fungi mediated the moderate reductions in sporulation of *C. rosea* and *B. cinerea* associated with the preinoculation humid period. Based on observations of conidiophores, densities of indigenous fungi increased markedly when senescent and dead leaves were kept in high humidity for 24 h. While not measured, bacteria also might have increased. Elevated densities of indigenous microbes potentially suppressed colonization and sporulation of *C. rosea* and *B. cinerea* through intensified substrate competition or other modes of antagonism within the tissues. Although sporulation was reduced, germination of *C. rosea* and *B. cinerea* on the phylloplane were not affected, at least when each fungus was inoculated alone. Notwithstanding the moderate suppression of *B. cinerea* conidiophores associated with the preinoculation humid period, few or no conidiophores of the pathogen were produced in leaves that were inoculated with *C. rosea* plus the pathogen, regardless of preinoculation high humidity and promotion of indigenous microbes.

Findings that the isolates of indigenous fungi suppressed germination and sporulation of *B. cinerea* in rose leaves were consistent with earlier observations in rose and other hosts (Redmond et al., 1987; Peng and Sutton, 1991; Sutton et al., 1997; Tatagiba et al., 1998). However, important interactions were found between these isolates, *C. rosea* and *B. cinerea*. For example, *A. alternata*, alone or in combination with *B. cinerea*, reduced sporulation of *C. rosea*, as did *Aspergillus* plus *B. cinerea* and *Penicillium* plus *B. cinerea*. Sporulation of *B. cinerea* was reduced by 99% in leaves treated with *C. rosea* alone, but by only 83% in leaves treated with *C. rosea* plus *Penicillium* sp., and by 78% in those treated with the agent plus *A. alternata*. Thus while the indigenous microbes each potentially contributed to control of *B. cinerea*, high inoculum density of the microbes in combination with *C. rosea* provided less effective control than the agent alone. There was no indication, however, that natural densities

of indigenous microbes would seriously compromise the ability of the agent to control *B. cinerea* in rose foliage.

In conclusion, *C. rosea* exhibited high flexibility in associating with leaves and petals of rose, and in controlling sporulation of *B. cinerea* in leaf and petal residues. The agent established in the host and effectively suppressed the pathogen regardless of the developmental stage of the host organs and presence of minor tissue wounds, a finding that is relevant in roses injured by production practices or by pests such as aphids and mites (Morandi et al., 2000). Further, *C. rosea* functioned well as a control agent whether or not the rose tissues were subjected to humid conditions that increased the natural microflora, which included several cosmopolitan genera of fungi. Given the versatility of *C. rosea*, the agent can be expected to control sporulation of *B. cinerea* in rose production systems, provided that temperature and humid periods are favorable, and especially when targetted at both the living foliage and flowers, and crop residues. The role of *C. rosea* in controlling sporulation of *B. cinerea* generally is not fulfilled by available fungicides, which interfere chiefly with the infection process and generally are not effective against the pathogen in crop residues (Köhl and Fokkema, 1998).

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