Antagonism of the Two-Needle Pine Stem Rust Fungi *Cronartium flaccidum* and *Peridermium pini* by *Cladosporium tenuissimum* In Vitro and In Planta

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ABSTRACT

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Selected isolates of *Cladosporium tenuissimum* were tested for their ability to inhibit in vitro aeciospore germination of the two-needle pine stem rusts *Cronartium flaccidum* and *Peridermium pini* and to suppress disease development in planta. The antagonistic fungus displayed a number of disease-suppressive mechanisms. Aeciospore germination on water agar slides was reduced at 12, 18, and 24 h when a conidial suspension (1.5×10^7 conidia per ml) of the *Cladosporium tenuissimum* isolates was added. When the aeciospores were incubated in same-strength conidial suspensions for 1, 11, 21, and 31 days, viability was reduced at 20 and 4°C. Light and scanning electron microscopy showed that rust spores were directly parasitized by *Cladosporium tenuissimum* and that the antagonist had evolved several strategies to breach the spore

wall and gain access to the underlying tissues. Penetration occurred with or without appressoria. The hyperparasite exerted a mechanical force to destroy the spore structures (spinules, cell wall) by direct contact, penetrated the aeciospores and subsequently proliferated within them. However, an enzymatic action could also be involved. This was shown by the dissolution of the host cell wall that comes in contact with the mycelium of the mycoparasite, by the lack of indentation in the host wall at the contact site, and by the minimal swelling at the infecting hyphal tip. Culture filtrates of the hyperparasite inhibited germination of rust propagules. A compound purified from the filtrates was characterized by chemical and spectroscopic analysis as cladosporol, a known β -1,3-glucan biosynthesis inhibitor. Conidia of *Cladosporium tenuissimum* reduced rust development on new infected pine seedlings over 2 years under greenhouse conditions. Because the fungus is an aggressive mycoparasite, produces fungicidal metabolites, and can survive and multiply in forest ecosystems without rusts, it seems a promising agent for the biological control of pine stem rusts in Europe.

Though climatic conditions favorable for severe rust epidemics occur every several years, the native stem rusts of two-needle hard pines, *Cronartium flaccidum* (Alb. et Schwein.) G. Winter and *Peridermium pini* (Pers.) Lév. (*Endocronartium pini* [Pers.] Y. Hirats.) (13,18,27), in Europe represent a constant and serious menace to Scots pine (*Pinus sylvestris* L.) in the temperate regions, and to Aleppo pine (*Pinus halepensis* Mill.), Austrian pine (*Pinus nigra* Arn.), Laricio pine (*Pinus laricio* Poir.), Italian stone pine (*Pinus pinea* L.), and Maritime pine (*Pinus pinaster* Ait.) in the Mediterranean region. These rusts cause major losses in pine production and are among the most common and destructive diseases of hard pines in the Northern Hemisphere. The heteromacrocyclic *Cronartium flaccidum* occurs throughout Eurasia, from near sea level to the timberline, on a number of indigenous pine host species with several herbaceous plants as its alternate hosts (13,39). The endocyclic *Peridermium pini*, derived by reduction from the closely related heteroecious rust, has a geographic distribution that overlaps with the huge range of *Cronartium flaccidum* in some areas. However, it does not require an alternate host to complete its life cycle, but passes directly from pine to pine, forming perennial infections on the branches and stems of susceptible hosts (58). This allows rapid intensification of the disease when conditions for infection are optimal.

A number of strategies have been adopted to counter pine rusts (16). Silvicultural practices, such as pruning of the lower branches

to reduce inoculum sources and eradication of alternate hosts in and around pine stands, have always led to inconclusive results. Chemical treatment to eliminate these alternate hosts appears to be both environmentally unacceptable and economically unfeasible (24). Such approaches would in any case be ineffective with the plurivorous *Cronartium flaccidum* because of the great number of its alternate hosts and the very wide distribution of the main alternate host, *Vincetoxicum hirundinaria* Med. (35). Likewise, protecting the pines against a short-cycled rust such as *Peridermium pini* is obviously impossible, because it is restricted to pine and lacks alternate hosts that could be removed. Therefore, the most effective control strategy for pine stem rust fungi seems to be the selection and breeding of rust-resistant pines (14,20,26,42). Generally, however, breeding programs for resistance to the native *Cronartium flaccidum* and *Peridermium pini* have not been successful (43), and this has prompted a demand for alternative control strategies.

Considerable interest has recently been aroused by the prospect of exploiting mycoparasitism for the biological control of plant diseases. Many studies have been conducted on the antagonistic relationships between parasitic fungi and host fungi, both in vitro and in planta. Because of practical constraints, it is difficult to obtain conclusive evidence that hyperparasites can protect plants against diseases under natural conditions, but some successful field studies have been reported (15,25,28,46).

A number of fungi have been found in association with the conifer rusts, but the nature of these associations is complex and poorly understood. They may be true parasites on rust fungi, or common saprophytes, or unrelated parasites that compete with the obligate parasites for host tissue colonization (5,21,36,40,41).

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Though the identity of most of the mycoflora associated with rust fructifications is still unknown, a number of nonrust fungi have been identified and investigated as potential biocontrol agents. Among these are several *Cladosporium* spp., which are aggressive hyperparasites that parasitize the various spore states of the rust fungi (3,17,23,31,49,52,53,55).

The rust hyperparasite *Cladosporium tenuissimum* Cooke was first reported to be parasitic against uredospores of *Melampsora larici-populina* Kleb. on *Populus × euroamericana* in Australia (50,51). This dematiaceous Hyphomycete has recently also been identified from field-collected aeciospore samples of *Cronartium flaccidum* and *Peridermium pini* in Europe (34). Many factors suggest that this fungus merits consideration as a potential biocontrol agent: (i) its reported antagonistic capabilities towards *M. larici-populina*; (ii) its close resemblance to *Cladosporium uredinicola* Speg., a common colonizer of telial columns of the fusiform rust *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (on leaves of *Quercus alba* L., *Q. lyrata* Walt, and *Q. nigra* L.) and other rusts (33); (iii) its cosmopolitan nature (9); and (iv) the high incidence of its airborne spores disseminated into the environment in the hottest months of the year (1), a period that coincides with maturation of aecial, uredial, and telial stages of pine rusts.

The objectives of the present study were (i) to test the ability of *Cladosporium tenuissimum* to colonize rust spores in vitro and suppress spore germination; (ii) to study the fine structure of the host–parasite interface and elucidate the basic principles underlying parasitism; (iii) to examine whether the mycoparasite produces any toxic substances; and (iv) to determine the effectiveness of *Cladosporium tenuissimum* in controlling or reducing rust disease in planta under greenhouse conditions.

MATERIALS AND METHODS

Organisms. Aeciospore samples were collected at various European sites in May, June, and July depending on latitude and altitude above sea level over a 3-year period (1996 to 1998), from pustules of rust-infected pine shoots and stems (Table 1). Inoculum was collected with a cyclone collector, which draws in spores and automatically deposits them in small gel capsules, or was scraped from the aecia with a sterile spatula and placed in 0.5-ml Eppendorf tubes. The capsules or tubes were wrapped in aluminum foil to avoid light damage, maintained for a week in a desiccator containing dried silica gel at 4°C to remove excess moisture, and stored at –20°C until use. Following rehydration for 24 h at room temperature, the inoculum from each rust provenance was seeded on 60-mm diameter plastic petri dishes filled with 10 ml of potato dextrose agar (PDA, Difco

Laboratories, Detroit) and incubated at 21°C in the dark to obtain growth of contaminants. The rusts did not grow on PDA, but colonies of several nonrust fungi started developing after a few days of incubation.

Rust-associated fungi were isolated in purity and identified as belonging to various genera, the more common being *Alternaria*, *Aspergillus*, *Aureobasidium*, *Penicillium*, and *Cladosporium*. The most frequent genus was *Cladosporium*, three species of which were identified: *Cladosporium cladosporioides* (Fresen.) de Vries, *Cladosporium herbarum* (Pers.: Fr.) Link, and *Cladosporium tenuissimum* Cooke. The identification of these fungi was confirmed by the Centraalbureau voor Schimmelcultures (CBS), Baarn, the Netherlands. *Cladosporium tenuissimum*, already recorded as a destructive rust mycoparasite and isolated from rust pustules with very high frequency, was examined for possible antagonism to two-needle pine rusts. The congeneric species, known to be ubiquitous, plurivorous saprophytes, and the contaminants from the other genera were not considered in the present study.

The DNA from the first authenticated isolate of *Cladosporium tenuissimum* (Itt₂₁) (Table 1) was extracted and the internal transcribed spacer (ITS) region, which occurs between the regions coding for nuclear small- and nuclear large-subunit rRNA, was amplified by polymerase chain reaction (PCR) with the general ITS1–4 primers (59). A 573-bp sequence, including the internal 5.8S gene and portions of the adjacent ITS1–2 spacers, was determined (EMBL accession no. Y15966) (34). All the other *Cladosporium* isolates were subsequently identified over the 3-year collection period by conventional means. ITS sequence comparison with the Itt₂₁ isolate allowed conclusive identification.

In vitro antagonism assays. The antagonistic capabilities of *Cladosporium tenuissimum* were tested on solid and liquid media under various conditions. One antagonistic isolate from each country was tested on a rust isolate from a provenance of that country. Four disjunct rust provenances Altyre (Al), Monticiano (Mc), Mount Pieria (Mp), and Thetford (Th), and four antagonistic isolates, Sc₁, Itt₂₁, Gr₄, and En₁, derived from those same provenances, were utilized in all the antagonism tests. Prior to each experiment, the viability of the rust propagules was assessed on subsamples of 24-h rehydrated aeciospores.

Interaction between *Cladosporium tenuissimum* and rust fungi on solid medium. Sterilized glass slides covered with a thin film of 2% water agar were inoculated with aeciospores (5 mg) from each rust provenance and with 25-μl aliquots of conidial suspensions (1.5×10^7 conidia per ml of distilled water) of the selected isolates of *Cladosporium tenuissimum*. To determine the optimum antagonistic effect, three deposition sequences were used in the incubation tests (38): (i) inoculation of the parasite 1 h

TABLE 1. Aeciospore samples from provenances of the two-needle pine rusts *Cronartium flaccidum* and *Peridermium pini* tested for the presence of the hyperparasite *Cladosporium tenuissimum*

Fungus	Host pine	Location	Isolate code	Region	Country	Hyperparasite isolation	Hyperparasite culture code ²	Supplier
<i>Cronartium flaccidum</i>	<i>Pinus sylvestris</i>	Naz	Nz	Trentino A. Adige	Italy	+	Ittr10	A. Dal Col
<i>Cronartium flaccidum</i>	<i>Pinus pinea</i>	San Vincenzo	Sv	Tuscany	Italy	–	Itt12	A. Ragazzi
<i>Cronartium flaccidum</i>	<i>Pinus pinaster</i>	Monticiano	Mc	Tuscany	Italy	+	Itt21	S. Moricca
<i>Cronartium flaccidum</i>	<i>Pinus nigra</i>	Monte Vosgi	Mv	Tuscany	Italy	–	Itt45	S. Moricca
<i>Cronartium flaccidum</i>	<i>Pinus halepensis</i>	Mercadante	Md	Apulia	Italy	+	Itap2	N. Luisi
<i>Cronartium flaccidum</i>	<i>Pinus halepensis</i>	Pulicchie	Pu	Apulia	Italy	–	–	N. Luisi
<i>Cronartium flaccidum</i>	<i>Pinus laricio</i>	Serra delle Giumente	Gi	Campania	Italy	+	Itca20	S. Moricca
<i>Cronartium flaccidum</i>	<i>Pinus laricio</i>	San Gregorio	Sg	Campania	Italy	–	Itca7	S. Moricca
<i>Peridermium pini</i>	<i>Pinus sylvestris</i>	Thetford	Th	East Anglia	England	+	En1	K. Mitchelson
<i>Peridermium pini</i>	<i>Pinus sylvestris</i>	Jokela	Jo	Rovaniemi	Finland	–	–	J. Kaitera
<i>Peridermium pini</i>	<i>Pinus sylvestris</i>	Iinattijärvi	Ii	Pudasjärvi	Finland	–	–	J. Kaitera
<i>Peridermium pini</i>	<i>Pinus sylvestris</i>	Mount Pieria	Mp	Macedonia	Greece	+	Gr4	S. Diamandis
<i>Peridermium pini</i>	<i>Pinus sylvestris</i>	Altyre	Al	Morayshire	Scotland	+	Sc1	K. Mitchelson

² S. Moricca private culture, held at the IPAF (Institute of Forest Tree Pathology, Florence, Italy). Numbers designating the individual antagonistic isolates are printed as subscripts in the text.

before the mycoparasite (deposition sequence I); (ii) inoculation of the parasite 1 h after the mycoparasite (deposition sequence II); and (iii) inoculation of parasite and mycoparasite simultaneously (deposition sequence III). The inoculated slides were incubated in moist chambers in the dark at $20 \pm 1^\circ\text{C}$, and aeciospore germination was observed under a Zeiss Lab microscope ($\times 400$) at 12, 18, and 24 h after inoculation. As a control, rust aeciospores were inoculated under the same conditions with only 25 μl of sterile water. The experiment was repeated three times, with four replicates for each treatment in which each slide served as a replicate, and the data were pooled.

Effect of *Cladosporium tenuissimum* on the viability of rust spores in storage. Samples of 25 mg of aeciospores from each rust provenance were desiccated for 24 h at 5°C , then inoculated with 25 μl of a conidial suspension (1.5×10^7 conidia per ml of distilled water) of each mycoparasitic isolate and incubated at -20 , 4 , and 20°C . As controls, equal amounts of spores from each rust provenance were inoculated with only 25 μl of sterile water and incubated at the same three temperatures. The mixtures were put in 0.5-ml Eppendorf tubes, vortexed briefly, and incubated at one of the three temperatures. The viability of the aeciospores was assessed on subsamples of these mixtures after 1, 11, 21, and 31 days. For each viability sampling, a small amount (2 to 3 mg) of the mixture was removed from the tubes with a sterile dissecting needle, placed on sterilized glass slides covered with water agar and incubated in glass petri dishes (19-cm diameter) lined with water-saturated absorbent paper at the bottom. After incubation for approximately 5 h at room temperature (16 to 20°C), aeciospore germination was observed under the microscope ($\times 400$), with 0.5% KOH as the mounting medium. The experiment was conducted twice, with three replicates per treatment in which each tube served as a replicate, and the data were pooled.

Effect of *Cladosporium tenuissimum* culture filtrates on the aeciospore germination of rusts. The four antagonistic isolates Sc₁, Itt₂₁, Gr₄, and En₁ were grown in liquid stationary cultures of potato dextrose broth (PDB, Oxoid Ltd., Basingstoke, England) for 14 days at $20 \pm 1^\circ\text{C}$. The medium was separated from the mycelium by filtration with a Büchner funnel (Whatman No. 1 filter paper) followed by centrifugation at 15,000 g for 15 min. The supernatant was sterilized by filtration through 0.45- μm membrane filters (Millipore Corp., Bedford, MA). Aliquots of 25- μl culture filtrate from each isolate were then spread separately on sterilized water agar slides prepared as described previously. Each of the overlaid slides was then dusted with spores of one of the rust fungi, incubated in the dark at 22°C in moist petri dishes, and spore germination was recorded at 12, 18, and 24 h. As a control, uninoculated liquid PDB was spread on slides inoculated

with rust spores, and spore germination was tested after the same time intervals. The experiment was repeated three times, with four different culture filtrates of each antagonist strain serving as four replicates, and the data were pooled.

Secondary metabolite purification from *Cladosporium tenuissimum*. The four isolates of *Cladosporium tenuissimum*, Itt₂₁, Itap₂, Gr₄, and En₁, originating in different geographic areas (Table 1), were cultured on PDB as described previously. Culture filtrates and ground freeze-dried mycelia were extracted respectively in separating funnels with an ethylacetate/methanol (100:1) mixture and in Soxhlet apparatus, with hexane, ethylacetate, and methanol in succession. The same isolates were also cultured on PDA in Roux flasks for 14 days at 24°C . Large-scale secondary metabolite production was also performed for the isolate Itt₂₁ on PDA supplied with glycerol (8 ml/liter) and on MPGA (malt extract, peptone, glucose, agar 20-2-20 to 15 g/liter) in Roux flasks. All the cultures on agar media were extracted twice with 100 ml of ethylacetate/methanol (100:1). After evaporation of the organic solvents, the crude extracts were weighed separately (Table 2) and purified by silica gel column chromatography with a stepwise elution of dichloromethane/methanol. A main pure common metabolite was obtained from the 15:1 fraction and by further purification with silica gel PLC. This metabolite was chemically and spectroscopically analyzed by ^1H nuclear magnetic resonance (NMR), ^{13}C NMR, mass spectrometry, UV, and infrared.

Scanning electron microscopy of the host-parasite interaction. The host-parasite interface was investigated by scanning electron microscopy (SEM). Two replicates of 60-mm diameter plastic petri dishes with PDA were inoculated with rehydrated aeciospores from each rust provenance and with 25- μl aliquots of conidial suspensions of *Cladosporium tenuissimum* (1.5×10^7 conidia per ml). Control treatment consisted of two replicates of 60-mm diameter plastic petri dishes with PDA inoculated with the rust spores as described previously, plus 25- μl aliquots of sterile water. After incubation at $20 \pm 1^\circ\text{C}$ in the dark for 72 h, small squares of agar medium were cut out aseptically and prepared for SEM examination as follows. Samples were fixed in a 3% glutaraldehyde solution for 1 h, rinsed once in a 0.1 M phosphate buffer (pH 7.3) for 5 min, postfixed by immersion in 1% OsO₄ in a phosphate buffer (pH 7.3) at room temperature for 1 h, and rinsed three times in the same buffer for 5 min. The material was dried in a Bal-Tec 030 critical-point drier (Balzers Union Aktiengesellschaft, Balzers, Lichtenstein) with liquid CO₂. Samples were sputtered with gold palladium film with an S105A Edward sputter coater and examined with a Philips 505 SEM (Philips Gloeilampenfabrieken, Eindhoven, the Netherlands) at 20 kV.

TABLE 2. Crude extract of total secondary metabolites obtained from 14-day-old agar or broth cultures of four *Cladosporium tenuissimum* isolates, Itt₂₁, Itap₂, Gr₄, and En₁[†]

Isolate	Agar culture			Broth culture in PDB ^u			
	PDA ^v	PDA+gly ^w	MPGA ^x	Culture filtrate ^y	Mycelium ^z extracted with		
					Hex	EtOAc	MeOH
Itt21	13.8 (+)	22.4 (+)	32.0 (+)	1 (+)	15 (–)	23 (+)	73 (–)
Itap2	11.0 (+)	24.8 (+)	38.0 (+)	5 (+)	56 (–)	71 (+)	30 (–)
Gr4	14.0 (+)	25.7 (+)	34.1 (+)	3 (+)	27 (–)	23 (+)	80 (–)
En1	23.0 (+)	23.8 (+)	33.8 (+)	1 (+)	21 (–)	29 (+)	86 (–)

[†] Production is expressed as milligrams of weight from 100 ml of medium; (+) or (–) indicate presence or absence of cladosporel in the extracts, detected by thin-layer chromatography in benzene/diethyl ether/formic acid (1:3:0.5%, vol/vol), in comparison with the pure compound ($R_f = 0.6$).

^u Potato dextrose broth (Difco), 100 ml in 300-ml Erlenmeyer flasks.

^v Potato dextrose agar (Difco), 100 ml in 1-liter Roux flasks.

^w Glycerol added at 8 ml/liter PDA.

^x Malt extract-peptone-glucose-agar (20-2-20-15 g/liter).

^y Culture filtrates were washed with EtOAc, organic solvent dried on anhydrous Na₂SO₄ and evaporated.

^z Mycelium was separated from culture filtrates, freeze dried, ground, and extracted in Soxhlet apparatus with 200 ml of hexane, ethylacetate, and methanol in succession.

In planta antagonism assay. The spermatogonial stage of *Cronartium flaccidum* was inoculated with a mixture of conidia from the four *Cronartium tenuissimum* isolates Sc₁, Itt₂₁, Gr₄, and En₁ to evaluate the effectiveness of the mycoparasite in limiting sporulation of the rust. A mixture of these antagonistic isolates was used because (i) it more closely resembles a natural population of this cosmopolitan, airborne parasitic fungus and (ii)

no interaction between rust × *ladosporium* provenance was observed. The test was carried out on 2-year-old, rust-infected seedlings of Aleppo pine, Austrian pine, Italian stone pine, Laricio pine, Maritime pine, and Scots pine grown in 15 × 18 × 40 cm (height/width/length) plastic pots filled with universal loam and agriperlite. These seedlings, which had been inoculated the year before with basidiospores produced on infected leaves of the

Fig. 1. Effect of four *Cladosporium tenuissimum* isolates (En₁, Gr₄, Itt₂₁, and Sc₁) on the viability of aeciospores of *Cronartium flaccidum* and *Peridermium pini* in relation to the sequence of spore deposition. The deposition sequences were: I, aeciospores deposited and incubated for 1 h prior to deposition of conidia of the mycoparasite; II, conidia deposited and incubated for 1 h prior to deposition of aeciospores; and III, aeciospores and conidia deposited simultaneously. The percentage of germination of the four rust provenances A to C, Al, D to F, Mc, G to I, Mp, and J to L, Th, were tested at different time intervals (12, 18, and 24 h) after inoculation with conidial suspensions of the antagonist. For the controls, sterilized water was used instead of a conidial suspension. Values represent the average of 300 spore counts. Vertical bars are the standard errors of the mean.

alternate host, *V. hirundinaria*, were moistened with sterile water and sprayed with a suspension of the conidial mixture (1.5×10^7 conidia per ml). Control treatment consisted of rust-infected seedlings from the same species sprayed with sterile water/Tween 20. Each treatment consisted of six replicates, each replicate consisting of a pot containing 28 to 30 seedlings from one pine species. Replicate pots were incubated individually in polyethylene bags at 18 to $21 \pm 1^\circ\text{C}$ for 3 days. Treatments were then arranged in a randomized block design in a greenhouse chamber with a 12 h photoperiod (5,000 lux), 18 to $21 \pm 1^\circ\text{C}$, 45/70% relative humidity day/night.

At the start of the test, the seedlings from each pine species were counted and the amount of rust infection was calculated on the basis of a visual inspection as follows: (i) infections on the stem (presence or absence of visible signs of the pathogen); (ii) symptoms (degree of infection), rated as mild infection (no, or max 1 to 2 bark swellings on the stem; no, or max 1 to 2 pycnia on the stem) or severe infection (multiple bark swellings; multiple pycnia on the stem; dead top or dead twigs); (iii) percentage of infection, according to the formula $(a/b) \times 100$, in which a = number of infected seedlings per replicate, and b = total number of seedlings per replicate; and (iv) number of dead seedlings.

Disease evaluation was completed after 5 months. The antagonistic effect of the candidate mycoparasitic isolates was determined by calculating for each replicate the following values: (i) infections on the stem, determined as described previously; (ii) symptoms (degree of infection): mild infection (no, or max 1 to 2 bark swellings on the stem; no, or max 1 to 2 pycnia on the stem; no, or max 1 to 2 aecial pustules on the stem) or severe infection (multiple bark swellings on the stem; multiple pycnia on the stem; multiple aecial pustules on the stem; dead top or dead twigs); (iii) percent disease reduction, according to the formula $(a/b) \times 100$, in which a = number of seedlings developed into healthy plants, and b = number of infected seedlings at the start of the experiment; and (iv) percentage of dead seedlings, according to the formula $(a/b) \times 100$, in which a = number of dead seedlings, and b = total number of seedlings.

The experiment was performed twice, in 1999 and in 2000, each time on new plant material of the same age and species, but with the same inoculation technique and disease evaluation procedure.

Data analysis. All data were analyzed with the STATISTICA-Mac program (StatSoft, Tulsa, OK). Data were checked for conformity with the main assumptions of analysis of variance (ANOVA). Mean values for aeciospore germination, expressed as percentages, were separated with the least significant difference (LSD) test after arcsine transformation. The LSD test was also used to compare means of disease incidence in the greenhouse trials.

RESULTS

Antagonism of *Cladosporium tenuissimum* toward rust fungi on solid medium. The antagonistic effect of the *Cladosporium tenuissimum* isolates Sc₁, Itt₂₁, Gr₄, and En₁ on the germination of aeciospores for each sequence of spore deposition on the glass slides is shown in Figure 1. Conidial suspensions of all the antagonistic isolates caused a significant reduction in the mean percent germination of rust spores at 12, 18, and 24 h when compared with the controls (respectively 33, 39, and 46% versus controls, $P = 0.05$). ANOVA revealed that the sequence of deposition was the main factor causing variability in aeciospore germination, although incubation time and incubation time $\times$ deposition sequence interaction also significantly contributed to total variance ($P = 0.05$). Analyses of the data for each of the rust provenances showed no significant differences in the aeciospore viability. Similarly, the data for each of the *Cladosporium tenuissimum* isolates showed no significant differences, indicating

that all isolates were equally antagonistic to rusts from all provenances. Maximum inhibitory effect on aeciospore germination occurred when conidia were inoculated 1 h before the aeciospores (deposition sequence II). Inhibition was less marked when the conidia and the aeciospores were inoculated simultaneously (deposition sequence III), and was smaller still when conidia were inoculated 1 h after the aeciospores (deposition sequence I). Inhibition of rust spore germination was noted from the first inspection, after 12 h.

Survival of rust spores colonized by *Cladosporium tenuissimum* in storage. The viability of rust aeciospores stored in mixture with antagonistic conidia is illustrated in Figure 2. Compared with the controls, all the isolates of *Cladosporium tenuissimum* reduced the viability of the rust spores. The antagonistic isolates did not differ significantly in the degree to which they suppressed spore germination, nor were significant differences noted in the susceptibility of aeciospores among provenances. Aeciospore viability decreased consistently with increasing storage temperature. The antagonistic isolates displayed the greatest biocontrol activity at 20°C , when they strongly reduced spore germination from the first days of interaction (34% versus control, $P = 0.05$). In cold storage (4°C), the viability of the rust spores was reduced (58% versus control, $P = 0.05$), but it was still fairly high after 11 days (77% versus control, $P = 0.05$). However, storage under freezing conditions (-20°C) prevented the aeciospores from being parasitized by the mycoparasite, at least during the initial period of mixed storage. In all the antagonistic interactions, spore viability decreased with increasing duration of storage. Inhibition of spore viability was greatest after 31 days of incubation at 20°C , when it was only 6% versus control ($P = 0.05$).

Examination of spore germination under the light microscope provided an opportunity to explore some aspects of the inter-fungus parasitism. Rust aeciospores appeared heavily parasitized and overgrown by hyphae of the mycoparasite, with all spores near hyphae of *Cladosporium tenuissimum* ungerminated (Fig. 3A). Hyphae of the antagonist grew toward the rust spores, and in some cases adhered closely to a rust propagule, coiling around it and forming an enlarged appressorium-like structure (Fig. 3B). Adhesion to the aeciospore surface was frequently followed by penetration of the spore by the antagonist mycelium, which often grew through the rust spore and emerged on the other side (Fig. 3C).

Inhibition of rust spore germination by *Cladosporium tenuissimum*. Culture filtrates of *Cladosporium tenuissimum* isolates caused a dramatic reduction in aeciospore germination compared with the controls (Fig. 4). ANOVA of aeciospore germination after treatment with the antagonist culture filtrates detected that all the antagonistic isolates significantly reduced spore viability. The rust provenances, the antagonistic isolates, and the interaction term (rust provenance $\times$ antagonistic isolate) were not statistically significant factors ($P = 0.05$). No rust provenance was more susceptible than others to any isolate, nor was any antagonistic isolate more inhibitory than others to aeciospores from any rust provenance. The culture filtrate was toxic from the first inspection (after 12 h incubation), and remained fairly constant, though a slight and gradual increase in germination was noticed at subsequent inspections (18 and 24 h). Aeciospores from rust provenance Mc displayed reduced viability in both antagonist-treated samples and the untreated controls.

Light microscope examination of the rust propagules on glass slides treated with culture filtrate showed alterations in the morphology of the aeciospores, with some spinules detached from the spore surface and scattered (Fig. 5), suggesting possible lytic action.

Secondary metabolite production by *Cladosporium tenuissimum*. From all ethylacetate-soluble fractions of the four antagonistic isolates Itt₂₁, Itap₂, Gr₄, and En₁ cultivated on liquid and

Fig. 2. Percent germination of aeciospores of *Cronartium flaccidum* and *Peridermium pini* from four provenances (Al, Mc, Mp, and Th). Aeciospores were incubated at -20 , 4 , and 20°C , for 1 to 31 days, together with a conidial suspension of selected *Cladosporium tenuissimum* isolates A, En1, B, Gr4, C, Itt21, D, Sc1, or E, with sterile water as a control. Values represent the average of 300 spore counts.

solid media, a major pure common metabolite with the molecular formula $\text{C}_{20}\text{H}_{16}\text{O}_6$ was isolated, corresponding to molecular weight (MW) 352. Physicochemical and spectroscopic analysis of this compound and its triacetyl derivative were consistent with the structure of cladosporol, a metabolite first isolated from *Cladosporium cladosporioides* (47). It constituted up to 46% by weight of the crude extracts. The composition of the substrate affected the amount of cladosporol in the extracts. This was seen when glycerol was supplied to PDA or when MPGA, a rich culture medium containing organic nitrogen, was employed (Table 2). Moreover, the isolates differed in the amount of cladosporol produced; Itap₂ was the major producer. Cultures of Itap₂ in 40 Roux flasks with MPGA produced up to 700 mg of cladosporol, three times as much as the equivalent cultures on PDA. Cladosporol was detected mainly in the mycelium and was not secreted abundantly into the culture broth. It was not soluble in hexane, but could be efficiently extracted by ethylacetate. Crude extracts also contained other metabolites as minor fractions, and these are currently under investigation to determine their structure and biological activity.

Ultrastructural analysis of the host-parasite interaction.

Rust hyperparasitism by *Cladosporium tenuissimum* was clearly evident by SEM examination of the host-parasite interface. Growth of the antagonist was profuse and abundant in the vicinity of the rust aeciospores, with formation of hyphal bundles typical of many *Cladosporium* spp. (Fig. 6A) and the production of long, straight hyphae often coiled around the rust spores (Fig. 6B). The parasitic fungus appeared strongly attracted to the host and attached to the rust spore either by producing appressoria of various shapes and sizes, with which it set up an intimate relation with the spore (Fig. 6C to E), or by forming hyphal branches,

Fig. 3. Light micrographs showing close association of *Cladosporium tenuissimum* mycelium with rust aeciospores and their parasitization. A, Numerous hyphae of the hyperparasite near a group of ungerminated spores; bar = $20\text{ }\mu\text{m}$. B, A mycoparasite hypha adhering to the surface of a rust spore and coiling around it to form an enlarged body resembling a hand-shaped appressorium (arrow); bar = $20\text{ }\mu\text{m}$. C, A mycoparasite hypha adhering closely to (arrow) and growing through a rust aeciospore; bar = $20\text{ }\mu\text{m}$.

which became appressed to and penetrated adjacent spores (Fig. 6F). The initial event of parasitism was therefore the direct growth of the parasite hyphae onto the host surface and their adhesion to the rust spores. Adhesion to the host cell wall seemed to be facilitated by an abundant production of amorphous, fibrillar material at the host-parasite interface (Fig. 7A). However, penetration of spores without the formation of any infection structures was also common. In many cases, it appeared to be due to a simple mechanical process of physical pressure exerted against the host cell wall. Once the mycelium of the mycoparasite had penetrated into the cell, it proliferated within and above the host propagule, and a hypha of the mycoparasite often grew directly out of the invaded spore, bearing some spinules on its apex (Fig. 7B). In the vicinity of groups of spores an increase was observed in the diameter of the hyphae, which grew between the cells, producing a swollen structure. Constrictions at the site where these hyphae passed between and into the host spores caused displacement of the spinules on the surface (Fig. 7C). As a consequence of this intimate contact between host and parasite, many spores collapsed even without observable penetration, suggesting a degradation of the spore cell content (Fig. 7D). Direct penetration by the parasitic fungus without indentation of the host cell at the contact site, or any other sign of mechanical force, was also observed (Fig. 7E and F). In some cases the spore surface dissolved at the point where it was in contact with the hypha of the parasite; however, it did not show any signs of physical

deformation of the spinules (Fig. 7G). No degradation was observed in spores in the control treatment.

Interaction between *Cladosporium tenuissimum* and *Cronartium flaccidum* on *Pinus* seedlings. Data from artificial inoculations carried out in 1999 and in 2000 indicated that rust infection was much more severe in the untreated controls than in the pine seedlings inoculated with a conidial suspension of the myco-

Fig. 5. Light micrograph of aeciospores treated with culture filtrate of the antagonist. Note the presence of spinules detached from the spore surface and scattered (arrows); bar = 20 μ m.

Fig. 4. Percent germination of aeciospores of *Cronartium flaccidum* and *Peridermium pini* from four provenances, A, Al, B, Mc, C, Mp, and D, Th, at three time intervals (12, 18, and 24 h) after treatment with a culture filtrate of the four antagonistic *Cladosporium tenuissimum* isolates En₁, Gr₄, Itt₂₁, and Sc₁. As a control, uninoculated liquid potato dextrose broth was used as a medium instead of the culture filtrate. Values represent the average of 300 spore counts. Vertical bars are the standard errors of the mean.

parasite. By the end of the experiments, the number of infections on the stem was significantly reduced ($P = 0.05$) in antagonist-treated seedlings of Austrian pine, Laricio pine, and Scots pine, which did not present any visible pustules on the stem. In all species treated with the antagonist conidia, the degree of infection remained mild, whereas in the controls it became uniformly severe (Table 3). The percentage of infected seedlings was also significantly lower ($P = 0.05$) in the antagonist-treated seedlings compared with the untreated controls. The ANOVA of data for disease reduction demonstrated that, although the treatment with antagonist conidia was the major factor causing variability in disease incidence, the pine species was also a significant contributor to total variance ($P = 0.05$). The rate of reduction was significantly greater ($P = 0.05$) on Austrian pine, Laricio pine, and Scots pine than on the other species. Rust reduction percentages for these species, averaging 1999 and 2000 values, were respectively 58.1, 50.1, and 64.9%, although they were only 28.4, 31.7, and 21.4%, respectively, for Aleppo pine, Italian stone pine, and Maritime pine. Average disease reduction for all species was 42.4%. The experiments in 2000 essentially paralleled those in

1999; mean percent reductions of rust disease in plants sprayed with the conidial mixture of the antagonist ranged from 66.6 to 19.1% and from 63.2 to 24.9%, respectively (Table 3). In the controls, disease incidence did not go down, but went up in all cases. At the end of the experiments, percent mortality was significantly ($P = 0.05$) lower in seedlings with antagonist inoculation than in those without. Mortality of antagonist-treated seedlings was significantly ($P = 0.05$) higher in the Mediterranean pines (Aleppo pine, Italian stone pine, and Maritime pine) than in Austrian pine, Laricio pine, or Scots pine (Table 3). The ranking of pine species for decrease in seedling mortality paralleled that for disease reduction in both growing seasons: Scots pine > Austrian pine > Laricio pine. The remaining species exhibited low to moderate abilities to inhibit rust disease.

A felty, dark greenish-brown mycelium in some cases covered the few spermatial and aecial fructifications on the bark of seedlings sprayed with the conidial suspension of the antagonist. The typical sporulating structures of *Cladosporium tenuissimum* (long, erect conidiophores with subapical ramo-conidia and conidia of different shape and length) were observed under the

Fig. 6. Scanning electron micrographs showing hyperparasitism of rust aeciospores by *Cladosporium tenuissimum* in vitro. **A**, A group of rust spores heavily overgrown and parasitized by mycelium of the mycoparasite. The long, straight-to-slightly-curved hyphae and the typical hyphal bundles (hb) of the antagonist can be seen; bar = 100 μ m. **B**, rust spores parasitized by the mycelium of the hyperparasite, which grows appressed to the rust spores, coiling around them (arrows) and forming numerous appressoria (a); bar = 100 μ m. **C to E**, Attachment of mycoparasite hyphae to the host surface by means of appressorium-like bodies of various shapes and sizes, which increase the area of contact between the two organisms, securely fastening the parasite to the host. Following initial swelling into **C**, a dome-shaped (ds) or **D**, multilobed (ls) structure, appressoria become firmly attached to the spore surface (arrows); **E**, bar = 10 μ m. **F**, Penetration (arrow) of a spore by a short side branch of the antagonist with no evidence of indentation in the host wall at the contact site; bar = 10 μ m.

microscope after scraping off decayed fructifications with a sterile glass needle and streaking the picked-up mycelial fragments and propagules on glass slides mounted with lactic acid and cotton blue. No mycelial growth was noted on the bark of seedlings of the untreated controls.

DISCUSSION

The present study shows that *Cladosporium tenuissimum* is a potential biocontrol agent of the two-needle pine rusts *Cronartium flaccidum* and *Peridermium pini*. A variety of independent investigations has demonstrated the effectiveness of *Cladosporium tenuissimum* antagonism both in vitro and in planta.

All the antagonistic isolates significantly reduced the germination of aeciospores from all the rust provenances. The lack of significant interaction between the source of a *Cladosporium tenuissimum* isolate and any of the rust provenances seemed to rule out the occurrence of physiological specialization. Germination was most strongly reduced when rust aeciospores were placed

on the slides 1 h after conidia of the mycoparasite. This suggested that, in nature, antagonism would be most effective if the antagonist colonized the phylloplane early and maintained a high inoculum density there.

Antagonist conidial suspensions also strongly reduced rust aeciospore germination in storage. Though the suppression of germination was limited at -20°C , when the biological activity of the antagonist was restricted, it was very high at 20°C . Even at 4°C , however, there was considerable biocontrol activity. These findings suggest that the fungus is adapted to temperate as well as to cold climates, as is confirmed by its very extensive range. The fact that the antagonist was still viable following treatment at these low temperatures also suggests that it has potential to overwinter in nature on perennial rust infections, and thus to produce inoculum for biological rust control whenever environmental conditions permit sporulation.

The inhibition of aeciospore germination by culture filtrates of *Cladosporium tenuissimum* suggested that inhibitory compounds were involved. Morphological changes in spores treated with the

Fig. 7. Scanning electron micrographs showing hyperparasitism of rust aeciospores by *Cladosporium tenuissimum* in vitro. **A**, Rust spores heavily parasitized by mycoparasite hyphae, with production of fibrous adhesive material (arrows) at the host–parasite interface; bar = 10 μm . **B**, A swollen hypha of the antagonist that penetrates a rust spore, causing displacement of the spinules (arrow). A hypha grows out of a penetrated rust spore carrying some spinules on its apex, with production of amorphous, fibrillar material (arrow); bar = 10 μm . **C**, Rust spores parasitized by swollen hyphae of the antagonist. Note a hypha emerging from a penetrated spore (arrow) and another hypha causing by its passage displacement of the spinules on the spore surface (arrow); bar = 10 μm . **D**, Late stage of appressorium-mediated parasitism of a rust spore that has collapsed, indicating that the hyperparasite may have degraded its contents; bar = 10 μm . **E and F**, Direct penetration of rust spores without evidence of indentation of the host cell at the contact site (arrows) or any other sign of mechanical action; bar = 10 μm . **G**, Host spore surfaces with evident signs of dissolution at the contact site with the antagonist hypha (arrow); bar = 10 μm .

culture filtrates, such as the displacement and scattering of spinules from the spore surface (visible under the light microscope), were presumably the result of the lytic action of enzymes or toxic agents, secreted into the medium by the antagonistic isolates. The ultrastructural investigations suggested that enzymatic activity could also be responsible for rust cell wall lysis and cell emptying, and that there is a recognition signal between host and hyperparasite. The main compound characterized from crude extracts of *Cladosporium tenuissimum* isolates was cladosporol, which is a plant growth regulator and an active β -1,3-glucan biosynthesis inhibitor, responsible for hyphal malformation in *Phytophthora capsici* (30,47,48). Spore ornamentation lysis, observed in our microscopic examination, could be an expected effect of this biological activity, which in turn could be ascribed to the unstable and reactive epoxide moiety of the molecule. Other substances containing the same functional group have the same effect (2,12).

Culture filtrates from isolates of *Cladosporium tenuissimum* inhibited spore germination more strongly than conidial suspensions, due to the higher concentration of toxic metabolites or lytic enzymes in comparison with natural conditions. The crude extracts contained other metabolites related to cladosporol and they could contribute to the activity of the culture filtrates, because the cladosporol occurred only in traces, being mostly detained by the fungal cell. Further investigations are needed to clarify the mechanism of toxic action of all the metabolites produced by the antagonistic fungus, including their involvement in the hyperparasite action in vivo.

The recent identification of three 12-membered macrolides from the culture filtrate of an isolate of *Cladosporium tenuissimum* from soil (11), two of which were also produced by *Cladosporium cladosporioides* (19), besides the finding that cladosporol was first isolated from a *Cladosporium cladosporioides* isolate (48), confirm the importance of using chemical fungal taxonomy for the systematic identification of species (10).

The exceptionally low viability of aeciospores from the Mc provenance in this assay, in both the antagonist-treated and the antagonist-untreated samples, should probably be attributed to high humidity at the time of collection, which caused many spores to clump together and subsequently to become discolored. This impaired the rust propagules and reduced spore viability. However, the antagonist further reduced aeciospore germination in this process, as well.

Light microscope and SEM observations suggest that *Cladosporium tenuissimum* has a number of mechanisms to gain access to the host aeciospores: mechanical penetration as a result of physical pressure exerted on the host cell wall, with or without the production of appressoria; or enzymatic action, with the dissolution and digestion of the spore surface, but without any apparent mechanical action.

Mycoparasites have become adapted to colonizing other fungi as a source of nutrients, and penetration represents a crucial step in this process. The penetration of the host cell wall by the parasite is important because it is the first step in establishing the host-parasite relationship (29). *Cladosporium tenuissimum* has evolved diverse invasion strategies to overcome the host barriers, and this probably gives it an advantage here.

Parasitization of rust aeciospores was also accompanied by abundant secretion of a film of fibrous material. This phenomenon, already reported both in fungi-higher plant interactions (4,7,8,22,37) and in interfungus parasitism (6,56), is thought to contribute to hyphal adhesion and preparation of the infection court (32). A certain increase in the hyphal diameter in the vicinity of rust spore surfaces has also been observed. A similar hyphal thickening was reported for *Cladosporium fulvum* when penetrating into the stomata of its host (57). Other physiological changes, such as the secretion of enzymes and an increase in pressure inside the infection structures, assist the penetration process. All such mechanisms are also employed by *Cladosporium tenuissimum*, and they are not exclusive to this fungus: several other members of this same genus, *Cladosporium aecidiicola* Thüm., *Cladosporium gallicola* Sutton, *Cladosporium hemileiae* Steyaert, *Cladosporium sphaerospermum* Penzig, *Cladosporium uredinicola* Speg., and *Cladosporium uredinophilum* Speg., are also mycoparasites (3,17,23,31,52,53), and some have more than one mode of parasitism (53,54,55).

The experiments in the greenhouse provided evidence that *Cladosporium tenuissimum* inhibits *Cronartium flaccidum* in planta. By the end of the experiments, disease incidence and severity were significantly higher in the antagonist-untreated controls than in the antagonist-treated seedlings. The biocontrol effectiveness of *Cladosporium tenuissimum* also seemed to depend on the degree of susceptibility of pine species to *Cronartium flaccidum*. Disease suppression was lower on Aleppo pine, Italian stone pine, and Maritime pine than on Austrian pine, Laricio pine,

TABLE 3. Effect of treatment of pine seedlings with conidia of *Cladosporium tenuissimum* on suppression of pine stem rust^v

Year	Pine species ^w	Disease severity								Disease incidence ^x			
		Stem infection ^y				Symptoms ^z				% Disease reduction		% Seedling mortality	
		Start		End		Start		End		End		End	
		A	C	A	C	A	C	A	C	A	C	A	C
1999	Aleppo pine	+ ^y	+	+	+	S	S	M	S	24.9 e	0	3.3 b	13.3 b
	Austrian pine	+	+	–	+	M	M	M	S	58.3 b	0	0 c	6.9 d
	Italian stone pine	+	+	+	+	M	M	M	S	31.7 d	0	3.4 b	10.3 c
	Laricio pine	+	+	–	+	S	S	M	S	54.6 c	0	0 c	6.6 d
	Maritime pine	+	+	+	+	S	S	M	S	23.8 e	0	7.1 a	17.2 a
	Scots pine	+	+	–	+	M	M	M	S	63.2 a	0	0 c	3.3 e
2000	Aleppo pine	+	+	+	+	S	S	M	S	31.9 d	0	6.6 b	13.7 b
	Austrian pine	+	+	–	+	M	M	M	S	57.9 b	0	3.3 c	10.3 c
	Italian stone pine	+	+	+	+	S	S	M	S	31.8 d	0	6.9 b	13.3 b
	Laricio pine	+	+	–	+	S	S	M	S	45.6 c	0	3.4 c	10.3 c
	Maritime pine	+	+	+	+	S	S	M	S	19.1 e	0	10 a	21.4 a
	Scots pine	+	+	–	+	M	M	M	S	66.6 a	0	0 d	3.4 d

^v Two-year-old, rust-infected seedlings were artificially inoculated under greenhouse conditions with a conidial mixture (1.5×10^7 conidia per ml) of antagonistic isolates Sc₁, Itt₂₁, Gr₄, and En₁ and surveyed for symptoms at the start and the end of the trials. The experiment was done in 1999 and in 2000, each year on new plant material.

^w A total of 28 to 30 seedlings (variation being due to seedling mortality prior to start of the trials) per species per treatment was used for each experiment.

^x For each year's trial, means in the same column not followed by the same letter are significantly different at $P = 0.05$, based on Fisher's protected LSD test.

^y A = antagonist-inoculated; C = no antagonist (control). + = infection on the stem (presence of pycnia and/or aecia); – = no infection on the stem.

^z S = severe infection (multiple bark swellings; multiple pycnia and aecial pustules on the stem; dead top or dead twigs); M = mild infection (no, or max 1 to 2 bark swellings on the stem; no, or max 1 to 2 pycnia on the stem).

and Scots pine. Previous studies demonstrated that the latter three species, Scots pine in particular, are less susceptible to rust colonization (43,45). These results are confirmed here. Among the mechanisms described in the previous studies as causes of the reduced colonization in resistant host pines are the biochemical inhibition of pathogen growth, and the formation of histological barriers preventing the progress of pathogen mycelium. Disease assessment was based on the degree of symptom development and disease incidence, which in turn were directly related to the extent of host tissue colonization by the rust pathogen. It followed that the ability of each individual species to mobilize biochemical and structural defenses was one of the factors in overall disease suppression, though the action of the mycoparasite remained, of course, the most important element.

Observation of a felty, greenish-brown mycelium on the spermatial sporulations, and subsequently on the few aecial sori that were produced, indicated that the hyperparasite also colonized rust fructifications, becoming established and producing abundant conidiophores and conidia on these structures. This was confirmed by microscopic examination of the hyperparasite's typical morphological features.

Cladosporium tenuissimum possesses many desirable characteristics of a biocontrol agent (51). It is adapted to many habitats (*Cladosporium tenuissimum* conidia are very common in collections of aeciospores from various countries), and a wide range of temperatures. Its unspecialized nature allows it to obtain nutrients from many substrates (9), and thus to survive, sporulate, and introduce enormous amounts of airborne inoculum into the environment. Moreover, *Cladosporium* spore production follows a seasonal cycle that peaks in the summer (1), precisely when the aecia, uredia, and telia of the host rust mature. Because of its aggressiveness, its cosmopolitan nature, and its epidemiological significance—chiefly on account of the details of its life cycle—*Cladosporium tenuissimum* would be expected to play an important role in the control of rust fungi. Indeed, it has been conjectured that parasitism by this fungus significantly reduced the severity of epidemics of *M. larici-populina* on *Populus × euroamericana* in the Canberra district of Australia for several years (50).

In Italy, disease surveys over the past few decades have revealed that the incidence of the rust caused by *Cronartium flaccidum* has recently been very variable (44). This has been attributed mainly to high temperatures, an unusually dry atmosphere, or other adverse conditions prevailing through most of the critical spring-to-summer period, when the rust fungus matures (35). However, recent surveys of some of the major rust foci have shown that disease incidence can be low even in years when the weather would be sufficiently humid and rainy for an outbreak. The mycoparasite is often associated with rust spores in these areas with otherwise unpredictable variations in rust incidence. From this we make the following deduction: in addition to the climatic factors that strongly influence the maturation of the spore states, the phylloplane mycoflora, and particularly *Cladosporium tenuissimum*, may also contribute to keeping disease incidence down.

By suppressing the production of rust spores, *Cladosporium tenuissimum* restricts rust damage and reduces the amount of rust inoculum introduced into the environment. The impact and significance of this action are very different for the two pine stem rusts, because they have different life cycles. In *Peridermium pini*, any reduction in the number of viable spores directly affects inoculum potential, but the full-cyclic *Cronartium flaccidum* can produce more inoculum on an alternate host. However, the great plasticity and ecological amplitude of *Cladosporium tenuissimum*, with its reported ability to colonize uredia of *M. larici-populina*, suggest that other spore stages of *Cronartium flaccidum* might also be parasitized, negating the advantage of greater inoculum production on other hosts.

Whether the mycoparasite is used to control the macrocyclic rust only on hard pines, or also on the dicotyledonous hosts, there is no doubt that the survival of rust propagules is greatly affected. A reduction in the number and longevity of the rust's survival structures could have consequences for the genetic structure of rust populations and the epidemiology of the disease. Investigations are required to better ascertain the role of *Cladosporium tenuissimum* in nature, and to assess its potential as a biocontrol agent. The studies detailed here indicate that the mycoparasite holds great promise for this purpose.

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LITERATURE CITED

1. Ainsworth, G. C. 1952. The incidence of air-borne *Cladosporium* spores in London region. J. Gen. Microbiol. 7:358-361.
2. Arnone, A., Nasini, G., Merlini, L., and Assante, G. 1986. Secondary mould metabolites. XVI. Stemphyliotoxins, new reduced perylenequinone metabolites from *Stemphylium botryosum* var. *lactucum*. J. Chem. Soc. Perkin Trans. 1:525-530.
3. Bolland, L. 1973. Poplar rust in Queensland. Aust. Plant Pathol. Soc. Newsl. 2:28.
4. Braun, E. J., and Howard, R. J. 1994. Adhesion of *Cochliobolus heterostrophus* conidia and germlings to leaves and artificial surfaces. Exp. Mycol. 18:211-220.
5. Byler, J. W., and Cobb, F. W., Jr. 1969. Fungi associated with galls caused by *Peridermium harknessii*. (Abstr.) Phytopathology 59:1020.
6. Carling, D. E., Brown, M. F., and Millikan, D. F. 1976. Ultrastructural examination of the *Puccinia graminis-Darluca filum* host-parasite relationship. Phytopathology 66:419-422.
7. Carver, T. L. W., Ingerson-Morris, S. M., Thomas, B. J., and Zeyen, R. J. 1995. Early interactions during powdery mildew infection. Can. J. Bot. 73:632-639.
8. Chaubal, R., Wilmot, V. A., and Wynn, W. K. 1991. Visualisation, adhesiveness and cytochemistry of the extracellular matrix produced by germ tubes of *Puccinia sorghi*. Can. J. Bot. 69:2044-2054.
9. Ellis, M. B. 1976. More Dematiaceous Hyphomycetes. Commonw. Mycol. Inst., Kew, Surrey.
10. Frisvad, J. C., Bridge, P. D., and Arora, D. K. 1998. Chemical Fungal Taxonomy. Marcel Dekker, Inc., New York.
11. Fujii, Y., Fukuda, A., Hamasaki, T., Ichimoto, I., and Nakajima, H. 1995. Twelve-membered lactones produced by *Cladosporium tenuissimum* and the plant growth retardant activity of cladospolide B. Phytochemistry 40:1443-1446.
12. Fukushima, Y., Sakagami, Y., and Marumo, S. 1993. β -glucan biosynthesis inhibitors isolated from fungi as hyphal malformation inducer. Bioorg. Med. Chem. Lett. 3:1219-1222.
13. Gümman, E. 1959. Die Rostpilze Mitteleuropas mit besonderer Berücksichtigung der Schweiz. Beitr. KryptogFlora Schweiz 12:1-407.
14. Gerhold, H. D., Goddard, R. E., and McDonald, G. I. 1986. Breeding out disease. J. For. 84:49-53.
15. Gerlagh, M., Goossen-van de Geijn, H. M., Fokkema, N. J., and Vereijken, P. F. G. 1999. Long-term bio-sanitation by application of *Coniothyrium minitans* on *Sclerotinia sclerotiorum*-infected crops. Phytopathology 89:141-147.
16. Hagle, S. K., McDonald, G. I., and Norby, E. A. 1989. White pine blister rust in northern Idaho and western Montana: Alternatives for integrated management. U. S. Dep. Agric. For. Serv., Intermt. Res. Stn. Gen. Tech. Rep. INT-261.
17. Heather, W. A., and Sharma, J. K. 1977. Some aspects of poplar rust research in Australia. Aust. For. 40:28-43.
18. Hiratsuka, Y. 1969. *Endocronartium*: A new genus for autoecious pine stem rusts. Can. J. Bot. 47:1493-1495.
19. Hirota, A., Sakai, H., and Isogai, A. 1985. Absolute stereochemistry of cladospolide A, a phytotoxic macrolide from *Cladosporium cladosporioides*. Agric. Biol. Chem. 49:903-904.
20. Hoff, R. J. 1986. Susceptibility of pine populations to western gall rust—

- central Idaho. USDA For. Serv., Intermt. For. Range Exp. Stn., Ogden, Utah. Res. Note INT-354.
21. Hubert, E. E. 1935. Some agencies attacking blister rust on white pine. J. For. 33:603-606.
 22. Jones, E. B. G. 1994. Fungal adhesion. Mycol. Res. 98:961-981.
 23. Keener, P. D. 1954. *Cladosporium aecidiicola* Thuem. and *Tubercolina persicina* (Ditm.) Sacc. associated with *Puccinia conspicua* (Arth.) Mains on *Helinium hoopesii* A. Gray in Arizona. Plant Dis. Rep. 38: 690-694.
 24. Ketcham, D. E., Wellner, C. A., and Evens, S. S., Jr. 1968. Western white pine management programs realigned on northern Rocky Mountain National Forests. J. For. 66:329-332.
 25. Köhl, J., Gerlagh, M., De Haas, B. H., and Krijger, M. G. 1998. Biological control of *Botrytis cinerea* in cyclamen with *Ulocladium atrum* and *Gliocladium roseum* under commercial growing conditions. Phytopathology 88:568-575.
 26. Kulhman, E. G., and Powers, H. R., Jr. 1988. Resistance responses in half-sib loblolly pine progenies after inoculation with *Cronartium quercuum* f. sp. *fusiforme*. Phytopathology 78:484-487.
 27. Kuprevicz, V. T., and Tranzschel, V. H. 1957. Flora plantarum kryptogamarum URSS. Vol. 4. Fungi (1). Uredinales Fasc. 1 Familia Melampsoraceae. Mosqua-Leningrad.
 28. Leibinger, W., Breuker, B., Hahn, M., and Medgen, K. 1997. Control of postharvest pathogens and colonization of the apple surface by antagonistic microorganisms in the field. Phytopathology 87:1103-1110.
 29. Manocha, M. S., and Lee, K. Y. 1971. Host-parasite relations in mycoparasites. I. Fine structure of host, parasite, and their interface. Can. J. Bot. 49:1677-1681.
 30. Marumo, S., Nakagawa, J., Chiba, N., and Yoshikawa, N. 1993. Plant growth regulator D101 manufacture with *Cladosporium*. Chem. Abstr. 119:269167.
 31. McKenzie, E. H. C., and Hudson, H. J. 1976. Mycoflora of rust-infected and non-infected plant material during decay. Trans. Br. Mycol. Soc. 66:223-238.
 32. Mendgen, K., Hahn, M., and Deising, H. 1996. Morphogenesis and mechanisms of penetration by plant pathogenic fungi. Annu. Rev. Phytopathol. 34:367-386.
 33. Morgan-Jones, G., and McKemy, J. M. 1990. Studies in the genus *Cladosporium sensu lato*: I. Concerning *Cladosporium uredinicola*, occurring on telial columns of *Cronartium quercuum* and other hosts. Mycotaxon 39:185-202.
 34. Moricca, S., Ragazzi, A., and Mitchelson, K. R. 1999. Molecular and conventional detection and identification of *Cladosporium tenuissimum* on two-needle pine rust aeciospores. Can. J. Bot. 77:339-347.
 35. Moriondo, F. 1975. Caratteristiche epidemiche della ruggine vescicolosa del pino, *Cronartium flaccidum* (Alb. et Schw.) Wint. In Italia. Annali Accad. Ital. Sci. For. 24:331-406.
 36. Myren, D. T. 1964. Insects and fungi associated with *Cronartium fusiforme*-infected tissue and comparisons of the strength of infected and healthy wood. (Abstr.) Phytopathology 54:902.
 37. Nicholson, R. L. 1996. Adhesion of fungal propagules. Pages 117-134 in: Histology, Ultrastructure and Molecular Cytology of Plant-Micro-Organism Interactions. M. Nicole and V. Gianinazzi-Pearson, eds. Kluwer Academic Publishers, Dordrecht, the Netherlands.
 38. Omar, M. B., and Heather, W. A. 1979. Effect of saprophytic phylloplane fungi on germination and development of *Melampsora larici-populina*. Trans. Br. Mycol. Soc. 72:225-231.
 39. Peterson, R. S. 1973. Studies of *Cronartium* (Uredinales). Rep. Tottori Mycol. Inst. 10:203-223.
 40. Peterson, R. S., and Jewell, F. F. 1968. Status of American stem rusts of pine. Annu. Rev. Phytopathol. 6:23-40.
 41. Powell, J. M. 1971. Fungi and bacteria associated with *Cronartium comandrae* on lodgepole pine in Alberta. Phytoprotection 52:45-51.
 42. Powers, H. R., Jr., and Kraus, J. F. 1983. Developing fusiform rust resistant loblolly and slash pines. Plant Dis. 67:187-189.
 43. Raddi, P., Mitterpergher, L., and Moriondo, F. 1979. Testing of *Pinus pinea* and *Pinus pinaster* progenies for resistance to *Cronartium flaccidum*. Phytopathology 69:679-681.
 44. Ragazzi, A. 1989. Past, present and future of *Cronartium flaccidum* in Italy. Phytopath. Medit. 28:5-9.
 45. Ragazzi, A., Fagnani, A., Dellavalle Fedi, I., and Mesturino, L. 1987. *Pinus pinaster* and *Pinus sylvestris*: Due specie a diverso comportamento verso *Cronartium flaccidum*. Phytopath. Medit. 26:81-84.
 46. Ragazzi, A., Moricca, S., Vagniluca, S., and Dellavalle, I. 1996. Antagonism of *Acremonium mucronatum* towards *Diplodia mutila* in tests *in vitro* and *in situ*. Eur. J. For. Pathol. 26:235-243.
 47. Sakagami, Y., Sano, A., Hara, O., Mikawa, T., and Marumo, S. 1995. Cladosporol, β -1,3-glucan biosynthesis inhibitor, isolated by the fungus *Cladosporium cladosporioides*. Tetrahedron Lett. 36:1469-1472.
 48. Sakagami, Y., Sano, A., Marumo, S., Yoshikawa, N., and Nakagawa, J. 1992. Cladosporol, a plant growth regulator produced by the fungus *Cladosporium cladosporioides*. Chem. Abstr. 120:238136.
 49. Sharma, J. K., and Heather, W. A. 1978. Parasitism of uredospores of *Melampsora larici-populina* Kleb. by *Cladosporium* sp. Eur. J. For. Pathol. 8:48-54.
 50. Sharma, J. K., and Heather, W. A. 1981. Antagonism by three species of *Cladosporium* to three races of *Melampsora larici-populina* Kleb. Aust. For. Res. 11:283-293.
 51. Sharma, J. K., and Heather, W. A. 1983. Post-penetration antagonism by *Cladosporium tenuissimum* to uredinial induction by *Melampsora larici-populina*. Trans. Br. Mycol. Soc. 80:373-374.
 52. Smith, R. E. 1905. Asparagus and asparagus rust in California. Calif. Agric. Exp. Stn. Bull. 165:1-99.
 53. Srivastava, A. K., Defago, G., and Kern, H. 1985. Hyperparasitism of *Puccinia horiana* and other microcyclic rusts. Phytopathol. Z. 114:73-78.
 54. Traquair, J. A., Meloche, R. B., Jarvis, W. R., and Baker, K. W. 1984. Hyperparasitism of *Puccinia violae* by *Cladosporium uredinicola*. Can. Bot. 62:181-184.
 55. Tsuneda, A., and Hiratsuka, Y. 1979. Mode of parasitism of a mycoparasite, *Cladosporium gallicola*, on western gall rust, *Endocronartium harknessii*. Can. J. Plant Pathol. 1:31-36.
 56. Tsuneda, A., and Skoropad, W. P. 1977. The *Alternaria brassicae*-*Nectria inventa* host-parasite interface. Can. J. Bot. 55:448-454.
 57. van den Ackerveken, G. F. J. M., Dunn, R. M., Cozijnsen, A. J., Vossen, J. P. M. J., van den Broek, H. W. J., and De Wit, P. G. M. 1994. Nitrogen limitation induces expression of the avirulence gene *avr9* in the tomato pathogen *Cladosporium fulvum*. Mol. Gen. Genet. 243:277-285.
 58. van der Kamp, B. J. 1968. *Peridermium pini* (Pers.) Lev. and the resin-top disease of Scots pine. I. A review of the literature. Forestry 41:190-198.
 59. White, T. J., Bruns, T., Lee, S. N., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols: A Guide to Methods and Applications. M. A. Innes, D. H. Gelfand, J. J. Sninski, and T. J. White, eds. Academic Press, New York.