19 Biocontrol of Rust Fungi by *Cladosporium tenuissimum*

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Introduction

The use of chemical pesticides in agriculture is under increasing public scrutiny, with mounting concern over possible harmful effects on the environment and on human health. Agrochemicals pollute groundwater, enter the food chain, have a deleterious impact on many living organisms and may bring about pesticide resistance in plant parasites. Increasing awareness of such possible hazards is prompting a heightened interest in alternative strategies (Boland and Kuykendall, 1998).

One of the most promising approaches to control economically important crop pests and diseases is by exploiting naturally occurring antagonists. Over the past few years unprecedented advances have been made in the biological control of many damaging insect pests and phytopathogenic microorganisms (Butt and Copping, 2000).

Many fungal pathogens throughout the world have natural enemies that limit the harm they cause. Some of these competitors are non-fungal hyperparasites such as bacteria (Yuen *et al.*, 2001) or mycoviruses (Brasier, 1990), but most are other fungi. Antagonistic fungi are a major component of the microbial community on the plant rhizosphere and phyllosphere, and play a pivotal role in regulating many interactions between plants and parasitic microorganisms (Jeffries, 1997). Since they are an integral part of the ecosystem, no alien microbe species, toxic substances or chemicals are introduced into the environment by their use, and hence they appear to be more environmentally sustainable than other, more intrusive, control methods. As a result, fungal biocontrol agents (BCAs) are becoming a promising means to control fungal diseases and to reduce dependence on chemical pesticides (Butt *et al.*, 2001).

However, developing a reliable and effective method for the biocontrol of a fungal pathogen is not a straightforward process. Several biological control experiments that were successful *in vitro* produced inconsistent results in the field. Moving from the laboratory or the greenhouse to large-scale field-testing is difficult because, in the field, the antagonist is subject to environmental influences. Plant disease is the result of a dynamic interaction over time between a pathogen, a plant and the environment. The environmental component of the disease triad is crucial for the success of a BCA, the limited ecological amplitude of

 Rust Diseases of Willow and Poplar (eds M.H. Pei and A.R. McCracken)

which might represent a major constraint. While an antagonist has to actively control a pathogen, a period that may take from a week to several months, it must be able to withstand fluctuations in the physical environment and resist the competing action of other, established microorganisms. The insufficient ecological fitness of the antagonist inoculum may lower its effectiveness as a control agent, while a poor shelf-life may cause lack of persistence, which means that the control achieved does not last over a sufficiently long term (Whipps and Lumsden, 2001).

This chapter examines whether *Cladosporium tenuissimum* Cooke, a destructive hyperparasite of rust spores, can be exploited as a BCA of rust fungi. It reports on tests carried out to evaluate *C. tenuissimum* effectiveness against rusts in the genera *Melampsora*, *Cronartium*, *Peridermium*, *Uromyces* and *Puccinia*, focusing on its modes of action, the fine-level analysis of the fungal host–hyperparasite interface, the antifungal compounds it produces, and its ability to reduce disease, both *in vitro* and *in planta*. Since effective biological control is impossible without due consideration of the ecology of the BCA, as well as of the other partners involved, and an examination of their spatial relationships and interaction with the environment, attention is also paid to the biological nature of the fungus, in particular to those characteristics that enable it to survive in natural habitats and retain activity under varying environmental conditions.

The Rust Disease Problem

The rust fungi (*Uredinales*) are one of the largest groups in the *Basidiomycota*, with about 5000–6000 species described on a wide range of hosts, including ferns, gymnosperms, and mono- and dicotyledonous angiosperms (Alexopoulos *et al.*, 1996). Rust fungi occur worldwide, under varied climates, and include destructive species that are responsible for costly diseases in agriculture and forestry.

Rust epidemics became particularly severe in the 20th century, after extensive monocultures had been aggressively established in agriculture and forests as a result of advances in plant genetics and the modernization of agriculture. But such developments ignored a basic principle in epidemiology, which is that both the number of diseases and disease incidence increase in proportion to host abundance. The advantage of identifying, planting, harvesting and marketing particular genetically homogeneous crops was therefore, in many cases, nullified by sudden disease outbreaks, caused directly by low crop diversity. The limitless potential for pathogen spread in monocultures led to the rapid selection of rust pathotypes able to overcome host defences. As a consequence, destructive fungi such as the coffee leaf rust *Hemileia vastatrix*, the wheat stem rust *Puccinia graminis*, the *Melampsora* leaf rusts of *Salicaceae* (*Populus* and *Salix*), the *Cronartium* stem rusts of hard pines, to name just a few, spread epidemically over vast areas, causing enormous losses and often making it necessary to replace susceptible crops entirely with non-host species (Littlefield, 1981).

At present, the main strategies to limit populations of rust pathogens and maintain yield stability are the continual breeding for new disease resistances and the search for new fungicides (to deal with the selection of new pathogen genotypes that attack resistant crops and that are resistant to existing fungicides), as well as crop rotation and the adoption of spatial and/or temporal crop diversity (Wolfe, 1985; Zhu *et al.*, 2000).

However, the effectiveness of such measures is subject to a number of limiting factors. Constant improvements in crop resistance and the never-ending need for new fungicides are costly to the farmer, the consumer and to society at large. Economic, environmental and technical reasons often make it impractical to use fungicides to combat rusts, especially rusts of forest trees. Other measures, such as the eradication of alternate hosts, scheduled harvesting (particularly of hazard-rated crops and stands), pop-up spacing, crop rotation with

non-hosts and standard sanitation procedures (removal of infected branches, burning of inoculum-bearing debris, etc.) are deficient because of the large geographic distribution of the pathogens, their wide host ranges, their high spore dispersal capabilities, and the autoecism of some rusts. Planting mixed genotypes with different disease-resistance profiles may be costly and may require particular expertise in cultivation practices, not to mention the fact that resistant plant varieties and fungicides are the major selective agents of pathogen virulence. Any single method of control thus has its limitations and it seems that a combination of measures in an integrated disease-management strategy is needed to counteract rust fungi.

Biological control is a non-chemical measure, exploiting microbiological agents to provide an additional method to combat rust disease. Among the common phylloplane mycobiota, many fungi have been reported since the first half of the 20th century, to be associated with rust fructifications (Arthur, 1929). Some of these fungi, eg. *Sphaerellopsis filum* (Biv.-Bern.:F) Sutton (teleomorph: *Eudarluca caricis* (Fr.) O. Erikss.), *Scytalidium uredinicola* Kuhlman *et al.*, *Aphanocladium album* (Preuss) W. Gams, and several species of *Cladosporium*, *Tuberculina* and *Verticillium*, have already demonstrated a pronounced hyperparasitism against different spore states of a number of rust fungi (Kuhlman *et al.*, 1978; Tsuneda *et al.*, 1980; Sharma and Heather, 1981a; Wicker, 1981; Allen, 1982).

Cladosporium Species Hyperparasitic on Rusts

The genus *Cladosporium* is one of the most widespread and prevalent genera of fungi, containing over 500 species Some of its species have teleomorphs in the ascomycete *Mycosphaerella* (*Dothideales*), but a vast majority of taxa are known by their anamorphic state or by criteria such as host association or presumed host specificity. These additional criteria are used to support the traditional classification because the inadequacy or lack of description for several members, and the phenotypic plasticity within individual taxonomic entities, make typification of species difficult or impossible. Substrate differences, climate and geographic variations also influence morphological expression. As a consequence, morphological features such as conidium size, shape, septation, pigmentation, surface characteristics (smooth or with ornamentation), conidiophore size and morphology are often variable and inconsistent (Morgan-Jones and McKemy, 1990; Ho *et al.*, 1999).

Members of this genus display a variety of lifestyles: some commonly occur on their hosts as epiphytes, endophytes, or as pathogens; others thrive as saprophytes, or even as hyperparasites (Ellis, 1976; Petrini, 1991; Moricca *et al.*, 1999; Dugan and Lupien, 2002; Larran *et al.*, 2002; Abdel-Baky and Abdel-Salam, 2003).

Several taxa in this large genus are prevalently associated with rust sori, and some are assumed to be invariably hyperparasites of *Uredinales* (Table 19.1). *Cladosporium uredinicola* Speg. is a common necrotrophic hyperparasite that destroys rust hyphae and causes coagulation and disintegration of the cell cytoplasm of a number of hosts, such as *Puccinia cestri* Dietel and P. Henn. (Spegazzini, 1912), *Puccinia recondita* Roberge ex Desm. (Ellis, 1976), *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (Morgan-Jones and McKemy, 1990), *Puccinia violae* (Schum.) DC (Traquair *et al.*, 1984) and *Puccinia horiana* (Srivastava *et al.*, 1985). Spegazzini (1922) reported that *Cladosporium uredinophilum* colonized and destroyed *Uredo cyclotrauma* Speg. propagules in Paraguay. Steyaert (1930) described *Cladosporium hemileiae* Steyaert as an effective hyperparasite of the coffee rust fungus *Hemileia vastatrix* in Zaire (Democratic Republic of Congo). Powell (1971) encountered *Cladosporium gallicola* in galls of *Cronartium comandrae* Pk. on *Pinus contorta* var. *latifolia* and considered the fungus parasitic on aeciospores and responsible for lowering aeciospore

production. Sutton (1973) reported a close association between *C. gallicola* and *Endocronartium harknessii* (J.P. Moore) Hiratsuka on *Pinus banksiana*, and observed hyphae of the hyperparasite penetrating the rust aeciospores. Tsuneda and Hiratsuka (1979) found that *C. gallicola* parasitized *E. harknessii* both by simple contact, disintegrating the cell walls of the spores, and by actual penetration of the spores walls, with or without the formation of appressoria, and causing the coagulation and disappearance of the host cytoplasm. These authors also found evidence that the rust spores, acting by contact, secreted enzymes in order to disintegrate the cell walls. A similar behaviour was noted for *Cladosporium aecidiicola*, a fairly common hyperparasite of rusts in Europe and in the Mediterranean area (Hulea, 1939; Rayss, 1943), parasitizing *E. harknessii* on *Pinus contorta*, *Pinus muricata* and *Pinus radiata* in California (Byler *et al.*, 1972). This hyperparasite also heavily parasitized aecia of *Puccinia conspicua* (Arth.) Mains. in Arizona (Keener, 1954) and urediniospores of *Melampsora medusae* Thüm under storage conditions (Sharma and Heather, 1980). Srivastava *et al.* (1985) found that *Puccinia horiana* was regularly parasitized by *Cladosporium sphaerospermum*. Sharma and Heather (1981a,b) reported that *C. tenuissimum* was a common phylloplane fungus that actively colonized urediniospores of the rust *Melampsora larici–populina* Kleb. on *Populus* × *euroamericana*. More recently *C. tenuissimum* was also detected in collections of aeciospores of the two-needle pine stem rust *Cronartium flaccidum* and its non-host alternating form *Peridermium pini* (Moricca and Ragazzi, 1998; Moricca *et al.*, 1999).

This list of *Cladosporium* species parasitic on rusts is not exhaustive and is likely to increase in the near future. The lack of genuine morphological structures and the great instability of such characters not only hamper the placement of *Cladosporium* species in a congruent evolutionary context, but also make it impossible to identify consistently many individual taxa and distinguish them from related ones. As new molecular techniques become increasingly employed to confirm and refine known taxonomic relationships, it is predictable that new taxa now undistinguishable or considered synonyms, will be added to the list. The number of hyperparasites is also expected to grow once it is shown that some *Cladosporium* species, now thought to be common saprophytes because they are found on aged host structures, are in fact true hyperparasites.

Table 19.1. *Cladosporium* species hyperparasitic on rust fungi.

Parasite	Host
C. aecidiicola Thüm	*Endocronartium harknessii* (J.P. Moore), (Byler *et al.*, 1972); *Melampsora medusae* Thüm (Sharma and Heather, 1980); *Puccinia conspicua* (Arth.) Mains., (Keener, 1954)
C. gallicola Sutton	*Endocronartium harknessii* (J.P. Moore) (Sutton, 1973; Tsuneda and Hiratsuka, 1979); *Cronartium comandrae* Pk. (Powell, 1971)
C. hemileiae Steyaert	*Hemileia vastatrix* Berk. et Br. (Steyaert, 1930)
C. sphaerospermum Penzig	*Puccinia horiana* Henn. (Srivastava *et al.*, 1985)
C. tenuissimum Cooke	*Melampsora larici–populina* (Sharma and Heather, 1978); *Cronartium flaccidum* (Alb. et Schwein.) G. Winter; *Peridermium pini* (Pers.) Lév. (Moricca *et al.*, 1999)
C. uredinicola Speg.	*Puccinia cestri* Dietel and P. Henn. (Spegazzini, 1912); *Puccinia recondita* Roberge ex Desm (Ellis, 1976); *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (Morgan-Jones and McKemy, 1990); *Puccinia violae* (Schum.) DC (Traquair *et al.*, 1984); *Puccinia horiana* (Srivastava *et al.*, 1985)
C. uredinophilum Speg.	*Uredo cyclotrauma* Speg. (Spegazzini, 1922)

Identification and Distribution of *Cladosporium tenuissimum*

The dematiaceous hyphomycete *Cladosporium tenuissimum* has long been known as a polyphagous saprophyte occurring in the air, on the soil and on plant surfaces (Ellis, 1976). It is a frequent colonizer of senescent or dead plant material and is common on the phyllosphere and rhizosphere of plant species, but it is also reported as an endophyte (Fisher and Petrini, 1992) and a facultative plant pathogen, causing blights, leaf spots, and seed, fruit and blossom-end rots (Pandey and Gupta, 1983; Xiang *et al*., 1989; Dohroo and Sharma, 1992; Sharma and Majumdar, 1993; Fujii *et al*., 1995; Dhal *et al*., 1997). Together with other *Moniliales*, it is the aetiological agent of human mycoses known as chromoblastomycosis, phaeohyphomycosis and eumycotic mycetoma (Gugnani and Okeke, 1989). The hyperparasitic nature of *C. tenuissimum* was first reported, as already mentioned, by Sharma and Heather (1981a,b) on the poplar rust *M. larici–populina* in Australia. It has recently attracted the attention of researchers because it suppresses rust under laboratory conditions and in glasshouses, and because it secretes compounds that are biologically active against various phytopathogens (Sharma and Heather, 1981a; Moricca *et al*., 2001; Assante *et al*., 2004).

The culture characteristics of this mitosporic fungus (colony appearance, texture and morphology, growth/temperature relationships, etc.), its micro-morphology (type of conidia and conidiogenesis) and other distinguishing features, are summarized in Moricca *et al*. (1999). The effect of nutrient composition on the production of secondary metabolites has also been investigated *in vitro* on different agar media and liquid cultures (Moricca *et al*., 2001). Irrespective of the culture medium, the fungus produces typical, geniculate and sympodially elongated conidiophores, by virtue of which it is unambiguously ascribed to the anamorph genus *Cladosporium* Link (Domsch and Gams, 1980).

Since the abundance of heterogeneous taxonomic elements makes traditional classification at species level difficult, representative European isolates of *C. tenuissimum* were identified by matching mycological characteristics with nucleotide sequences from coding and non-coding regions of the ribosomal RNA operon (Moricca *et al*., 1999) and by chemotaxonomic profiling (Moricca *et al*., 2001). Molecular differences in nucleic acid sequences were instrumental particularly in *vis-à-vis* species recognition, since congeneric, morphologically similar taxa (*C. herbarum*, *C. cladosporioides* and other unidentified *Cladosporia*) were syntopic to *C. tenuissimum*, often sharing the same ecological niche (rust sori).

Several *Cladosporium* members have not yet been comprehensively described, many of its taxa are still ill-defined and the appropriateness of their separate recognition remains a vexed question (Morgan-Jones and McKemy, 1990). Many species are not considered good species because they have not been identified with sufficient confidence. As a result, a number of ecological, phytopathological and biomedical studies identify *Cladosporia* only at the generic level (Bolland, 1973; McKenzie and Hudson, 1976; Heather and Sharma, 1977; Sharma and Heather, 1978; Hamada and Fujita, 2002; Chew *et al*., 2003). This means that information on the geographic distribution of *C. tenuissimum* in particular is scanty. Direct and indirect elements suggest, however, that it is an ubiquitous fungus with a worldwide distribution. It is known to be cosmopolitan and has been recovered from various matrices. In Europe it has been found on rust aeciospore samples of two-needled pines from various countries (Moricca *et al*., 2001). On the other hand, a high incidence of airborne *Cladosporium* inoculum was reported in the London region by Ainsworth (1952). More importantly, that author also found that *Cladosporium* inoculum reached a peak in summer, coinciding with maturation of most rust spore stages. The possibility for the hyperparasite to colonize spores and control their numbers at developmentally just the right time is particularly attractive in the

light of its potential exploitation for rust biocontrol

The Hyperparasitic Relationship

In vitro antagonism

Interaction on glass slides

The great potential of *C. tenuissimum* as a BCA has been evident since the initial experiments of Heather and Sharma (1977) and Sharma and Heather (1978, 1981a,b, 1983, 1988). These authors observed both direct parasitism of urediniospores of *M. larici–populina* by this hyperparasite, and inhibition of rust spores without any physical contact, suggesting that antibiosis might also occur. This evidence induced these researchers to postulate that *C. tenuissimum* had significantly reduced the incidence and severity of rust disease in poplar plantations in the Canberra district for several years (Sharma and Heather, 1981a).

The antagonistic capability of *C. tenuissimum* was further confirmed when it was found to inhibit, *in vitro*, the germination of propagules of other rust fungi. Selected isolates of *C. tenuissimum* significantly reduced average percentage germination of aeciospores of *C. flaccidum* and *P. pini* at 12, 18 and 24 h from inoculation with conidial suspensions of the hyperparasite (33, 39 and 46% versus controls, respectively) (Moricca *et al.*, 2001). The germination of urediniospores of the bean rust fungus *Uromyces appendiculatus* treated simultaneously with a conidial suspension of the antagonist isolate 'Itt21' was reduced significantly from 56.4% to 36.9% after just 3 h of contact, a reduction of 35%. After 6 h, urediniospore germination was still lower than in the control (69.5 versus 83.3%), and at the end of the observations the difference was 17% (Assante *et al.*, 2004). Beyond this time, *U. appendiculatus* urediniospores no longer germinated. Freshly collected aeciospores of the pine twist rust *Melampsora pinitorqua* and of the common rust *Puccinia sorghi* inoculated with a mixture of conidia from different antagonist isolates displayed, after 24 h, reductions in germination of 19% and 21%, respectively, compared with the controls (Torraca, Italy, personal communication).

Timing of infection

The reduction in spore germination might also depend on the time of initial infection. Experiments on *C. flaccidum* and *P. pini* indicated that the order in which the rust and hyperparasite were deposited was the main factor causing variability in aeciospore germination. In these experiments three deposition sequences were tested: (i) aeciospores deposited 1 h prior to the hyperparasite conidia; (ii) conidia deposited 1 h prior to the aeciospores; (iii) aeciospores and conidia deposited simultaneously. Maximum inhibition of aeciospore germination was achieved when conidia were inoculated 1 h before the rust aeciospores (Moricca *et al.*, 2001). This outcome suggests that, in nature, control is most effective if the antagonist establishes itself early on the host surfaces, before the rust, in order to build up a mass of inoculum sufficient to parasitize rust propagules as they burst from the plant epidermis somewhat later (Kranz, 1969a; Moricca *et al.*, 2001). However, the research data do not present a coherent picture. Other studies on *Puccinia recondita*, *Cronartium fusiforme* and *Cronartium strobelinum* found that with these rusts, infections became most severe when the antagonist *Darluca filum* was inoculated before them (Swendsrud and Calpouzos, 1972; Kuhlman *et al.*, 1978).

Interactions in storage

The recovery of *C. tenuissimum* at various latitudes, from separated geographic areas with varying climates, suggests that the fungus can survive and remain active under disparate environmental conditions. This adaptability is also shown by its ability to survive and parasitize aeciospores of *C. flaccidum* and *P. pini* in storage in a range of temperatures. In tests it was effective at

–20, 4 and 20°C. Control was greatest at 20°C but spore viability was decreased at all test temperatures, including –20°C. Viability also gradually decreased with storage time (Moricca *et al.*, 2001). Antagonist-treated spore lots were visibly discoloured. Stereoscope observations revealed deterioration of the rust propagules, which were densely intertwined with hyphae of the antagonist. The hyperparasite had proliferated extensively, giving rise to an appreciable mycelial biomass. Spore deterioration was the first indication that an exogenous enzymatic effect was probably involved in the disintegration process. The long shelf-life of *C. tenuissimum* inoculum at low temperatures provides evidence of ecological tolerance, a characteristic that is common to other hyperparasites (Kranz, 1969b). As in other fungal host–hyperparasite interactions, the antagonist thrives on the host spores, which represent an ideal *pabulum* (Swendsrud and Calpouzos, 1970). However, the host propagules are not vital for the antagonist which, being also a facultative saprophyte, a plant and animal parasite and an unspecialized hyperparasite, can survive on various materials such as plant debris, foliar exudates, small insects and other organic substances occurring in the environment.

Rust spores collected during adverse weather (high humidity, rainfall) and not properly dehydrated before storage, soon clumped together, and were impaired and discoloured. Many of these clumped spores were soon heavily overgrown with hyperparasite mycelium, which proliferated and sporulated profusely on them, markedly decreasing spore viability (Moricca *et al.*, 2001). High humidity is therefore favourable to infection, pathogenesis and sporulation of *C. tenuissimum*.

Effect of C. tenuissimum *culture filtrates*

Treatment of all rust spores with culture filtrates showed that enzyme(s) and/or toxic agent(s) had a role in *C. tenuissimum* parasitism. Aliquots (25 µl) of culture filtrates from four antagonist isolates spread separately on sterilized water-agar slides, dusted with aeciospores of two-needled pine rust fungi and incubated in the dark at 22°C in moist Petri dishes, strongly reduced spore germination after 12, 18 and 24 h, as compared with the controls. The rust provenances, the antagonist isolates and the interaction term (rust provenance × antagonist isolate) were not significant variables, indicating an absence of physiological specialization in the hyperparasite (Moricca *et al.*, 2001). Several of the spores examined individually under the microscope for viability were barely recognizable, with their spinules displaced and scattered all over the mounting medium, indicating an action of proteolytic enzyme(s) secreted into the medium.

The pronounced sterility of rust spores treated with *C. tenuissimum* culture filtrates suggested that the hyperparasite was a source of extracellular antifungal antibiotics, as it is in other hyperparasite–parasite interactions (Jackson *et al.*, 1997; Rodriguez and Pfender, 1997; Trejo-Estrada *et al.*, 1998). The toxicity of the culture filtrates was immediate, from the first inspection (after 12 h), and remained fairly constant for the duration of the experiment, with a slight increase in germination after 18 and 24 h.

Hyperparasitic secondary metabolites: a band of killers

C. tenuissimum actively produces metabolites with antifungal properties. These include a major pure common metabolite with the molecular formula $C_{20}H_{16}O_6$, and corresponding to M_r 352, and a series of related compounds, all of which were isolated from the ethylacetate crude extracts (EtOAc CEs) of several antagonist isolates (Moricca *et al.*, 2001). This metabolite was already known as cladosporol, a dimeric decaketide which had been isolated from *C. cladosporioides* (Fukushima *et al.*, 1993; Sakagami *et al.*, 1995). Cladosporol induces hyphal malformations in *Phytophthora capsici* when tested at 10 µg/disc (Fukushima *et al.*, 1993). It is also an inhibitor of β-1,3-glucan synthetase, the enzyme that synthesizes the fungal cell wall component β-1,3-glucan. In an *in vitro* assay with

labelled UDP-glucose and β-1,3-glucan synthetase prepared from *Saccharomyces cerevisiae*, cladosporol showed an IC_{50} activity on the enzyme at 50 μg/ml (Sakagami *et al.*, 1995).

The series of related compounds purified from EtOAc CEs of *C. tenuissimum* cultures, consists of cladosporols B, C, D and E (Assante *et al.*, 2002; Nasini *et al.*, 2004). The major metabolite, now named cladosporol A, was isolated as a white powder and represented more than 30% of the crude extract. A second metabolite had the same ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra as cladosporol A, except that it had a 4-oxo function instead of the C(4)HOH grouping. This metabolite was named cladosporol B. A third compound had the same basic skeleton as cladosporol A, but with a $C(2)H_2$-$C(3)H_2$ unit instead of the 2,3-oxirane ring. This compound was compatible with the molecular formula $C_{20}H_{18}O_5$ and was named cladosporol C. A fourth metabolite, cladosporol D, was a cream-coloured solid with the formula $C_{20}H_{18}O_6$. Its ^{1}H and ^{13}C NMR data, when compared with those of cladosporol C, indicated that it contained a C(3)HOH fragment instead of CH_2, the remaining signals being quite similar. The last metabolite, cladosporol E, was isolated as a brown solid with the formula $C_{20}H_{18}O_7$. Its ^{1}H and ^{13}C NMR spectra were very similar to those of cladosporol D, the only important difference being that this cladosporol had an additional hydroxy group at C-2 (Nasini *et al.*, 2004).

Cladosporols A–C, produced in an amount sufficient to enable some assays on their biological activity, inhibited, *in vitro*, a number of rust fungi, non-rust fungi, *Oomycota* and yeasts. They suppressed germination of urediniospores of *U. appendiculatus* and of aeciospores of *M. pinitorqua*, *C. flaccidum*, *P. recondita* and *P. sorghi*, in a range between 75 and 100%, when tested at 100 μg ml^{-1}. Cladosporol B was the most active of the group, completely suppressing germination of *U. appendiculatus* at 50 ppm, reducing it by more than 90% at 25 ppm, and lowering it even at 12.5 ppm. Cladosporol A, through less inhibitory than cladosporol B, was more active than cladosporol C, reaching an inhibition value higher than 80% at the highest concentration.

The cladosporols reduced radial growth of colonies of the phytopathogenic fungi *Alternaria alternata*, *Botrytis cinerea*, *Cercospora bieticola*, *Cercosporella herpotrichoides*, *Colletotrichum lindemuthianum*, *Fusarium roseum*, *Helminthosporium oryzae*, *Mucor* sp., *Rhizoctonia solani* and *Septoria tritici*; of the *Oomycota Phytophthora capsici*, *P. cinnamomi*, *P. erytroseptica*, *P. nicotianae* and *Pythium ultimum*; and of human-pathogenic strains of *Candida* sp. (Moricca *et al.*, 2001; Assante *et al.*, 2002; Nasini *et al.*, 2004; T. Kasuga, California, 2001, personal communication; Aloi and Fossati, Italy, 2002, personal communication). The strongest antagonistic effect was against the *Oomycota*.

Sensitivity of tested fungi varied in relation to concentration and differences in the functional groups bound in this family of metabolites to the tetralone skeleton. The antifungal activity of described cladosporols is likely to reside, as reported for other similar compounds (Arnone *et al.*, 1986; Fukushima *et al.*, 1993), in the intrinsic toxicity of the 2-tetralone chromophore and the occurrence of highly reactive substituents, like the epoxy group β-1,3-glucan, as a constituent of the fungal cell wall skeleton. By inhibiting the enzyme that synthesizes this constituent, the cladosporols directly affect the biochemistry and structural organization of the fungal cell. They thus strongly condition the pathogenicity of *C. tenuissimum* and play a major role in its hyperparasitism.

In vivo antagonism

In planta *assays*

Inoculation tests on whole rust-infected plants in a controlled environment (greenhouse or laboratory) give a first indication of how the rust may be controlled in nature. They therefore represent an important step in studying the mode of action of the hyperparasite. If a natural inoculation procedure

and an objective disease evaluation protocol are followed, *in planta* assays can, in just a few weeks, provide valuable data on the biocontrol effectiveness of a tested microorganism. Furthermore, in such an artificial system, the tri-trophic interaction between host plant, rust parasite and hyperparasite can be more accurately investigated, since the effect of the environment, which in the field can positively or negatively affect each interacting partner, is eliminated.

In two consecutive growing seasons, 1999 and 2000, spermogonia of *C. flaccidum* that had developed on 2-year-old, rust-infected pine seedlings, were inoculated with mixed conidial suspensions from different *C. tenuissimum* isolates. Rust-infected control seedlings were sprayed only with sterile water/Tween 20. Disease evaluation, based on a standardized procedure, was completed after 5 months, and the incidence and severity of the rust infection were defined. The percentage of infected seedlings and the number of infections per seedling stem were significantly lower in the antagonist-treated seedlings than in the untreated controls. Percentage mortality was significantly lower in seedlings with antagonist inoculation than in those without. A mycelial biomass attributable to the hyperparasite and detectable as a felty, dark greenish-brown mycelium was observed on spermatial and aecial fructifications on the bark of some treated seedlings. The fungus was positively identified by microscope examination of the sporulating structures (erect, straight, regularly septate conidiophores; holoblastic, conidiogenous cells; cylindrical to clavate ramo-conidia with 2–3 flattened, thickened scars; intercalary and terminal conidia of various shapes and sizes) (Moricca *et al.*, 2001).

In an experiment exploring the hyperparasitism of *C. tenuissimum* on *U. appendiculatus*, a classical disease escape mechanism may have prevented the antagonist from parasitizing the rust. Primary leaves of bean plants of *P. vulgaris* L. cv. 'Borlotto nano Lingua di fuoco' were inoculated on their lower surface with a suspension of *U. appendiculatus* urediniospore and simultaneously treated with a suspension of *C. tenuissimum* conidia, or with a MPGG (malt extract, peptone, glucose, glycerol) culture filtrate of a 2-week-old liquid stationary culture of the hyperparasite. Controls were healthy bean plants inoculated with: (i) a water suspension of *C. tenuissimum* conidia; (ii) rust urediniospores, then treated with sterile water/Tween 20; or (iii) only a sterile uninoculated MPGG broth. Disease severity was scored by the number of pustules per square centimetre in a total of eight 1-cm^2 leaf areas on digitalized primary leaves, after 13 d from inoculation. After 1 month, plants inoculated simultaneously with *U. appendiculatus* and the conidial suspension developed rust in the normal way. By contrast, treatment with the antagonist culture filtrate provided total protection: the urediniospores did not germinate and the bean plants did not develop any infection (Assante *et al.*, 2004).

A possible explanation of these findings on bean rust is that in the simultaneous infections the time available for inter-fungus interaction was too short to enable the antagonist to establish a parasitic relationship with the pathogen. The specialized, biotrophic agent found refuge by rapidly penetrating into the living host tissues, occupying the ecological niche it has evolved since primordial times to colonize in order to gain access to nutrients and protection from natural enemies. The antifungal compounds and enzymes in the culture filtrate, on the other hand, acted immediately and prevented propagule germination and rust development.

Detached leaves

A simple test in a strictly controlled environment, using Petri dishes and a water-saturated atmosphere, can give an insight into the type of hyperparasitic interaction. Primary leaves of bean rust-infected plants, inoculated as above with a conidial suspension or a culture filtrate of *C. tenuissimum*, were immediately detached and incubated in 15-cm diameter Petri dishes. The leaf stems were dipped in a medium containing 1% water-agar (WA) supplemented with

5 ppm gibberellic acid (GA). Hyperparasite colonization of rust pustules and disease severity were monitored daily under a stereoscope, starting 1 week after hyperparasite inoculation and continuing until the end of the experiment. Rust regularly formed appressoria at the precise location of the stomata (indirect-type, dikaryotic penetration) but these appressoria began to collapse a few hours after formation. In spite of the positive tropism of *C. tenuissimum* conidia towards the rust propagules, as indicated by the many conidia closely attached to rust spores and appressoria, penetration of the bean leaves by the rust could not be prevented completely, and this can explain why, compared with the controls, disease severity was curtailed by 13% only. As in the experiment with the whole plants, the culture filtrate had a toxic effect on the detached leaves that prevented any rust spores from germinating (Assante *et al.*, 2004).

LM, SEM and TEM Examination of the Host/Parasite Interface

Examination of the interface between *C. tenuissimum* and the rust agents *C. flaccidum*, *P. pini* and *U. appendiculatus* with light (LM), scanning (SEM) and transmission electron microscopy (TEM) showed the strong antagonistic action of the hyperparasite, and the multiple strategies it employed to parasitize the rust fungi. The reproductive capacity of *C. tenuissimum* appeared greatly enhanced by the proximity of rust spores, most of which were inactivated and overgrown by the antagonist mycelium. The hyperparasite sporulated profusely on the spores, producing on their surface tufts of fructifications bearing numbers of conidiophores. These conidiophores generated a multitude of asexual propagation units, represented by ellipsoidal to limoniform ramo-conidia, oblong or fusiform intercalary conidia, and mostly globose or subglobose terminal conidia. Prolific antagonist sporulation gave rise to an enormous mass of conidia which, together with the germ tubes and hyphae growing from them, were attracted to the rust propagules, to which they became firmly attached.

The contact stimulus between the host and the hyperparasite – a reflection of recognition events among them – mediated the secretion of different substances at the host–parasite interface. These substances are believed to play a fundamental role in pathogenesis, either in interactions between plants and parasitic fungi (Chaubal *et al.*, 1991; Braun and Howard, 1994; Jones, 1994; Carver *et al.*, 1995; Nicholson, 1996) or between those fungi and their hyperparasites (Carling *et al.*, 1976; Tsuneda and Skoropad, 1977; Moricca *et al.*, 2001). Some of these substances, visible as a dense network of amorphous, fibrous material, were the direct product of hyperparasite metabolism and served to ensure close adhesion of the hyperparasite to the host cell wall (Moricca *et al.*, 2001). Amorphous material from a different source was observed adjacent to shrunken and eroded parts of the spore wall. This second type of extracellular, amorphous material characteristically accompanied spore penetration and was associated with the hyperparasite structures involved in the process, i.e. the variously shaped appressoria formed on the host surface and the infection hyphae. Ultrastructural examination at contact points provided evidence that lytic enzymes caused degradation of the host cell walls and released the amorphous material at the rust–hyperparasite interface. A decrease in electron density from the outer to the inner layers of the spore wall sometimes made the fibrillar chitin structure of the wall visible (Assante *et al.*, 2004).

An adhesive matrix pad intimately connected the host and the hyperparasite to each other. This pad serves a double function, to attach and support the appressorium and the penetrating hypha, and to be a reservoir for enzymatic penetration. Such functions have already been reported both in inter-fungus parasitism and in host plant–parasite interactions (Gold and Mendgen, 1984, 1991; Benhamou and Chet, 1996; Askary *et al.*, 1997; Moricca *et al.*, 2001). The

growth of the antagonist on a synthetic medium containing the polymer laminarin as the sole carbon source, on the other hand, shows that it produces extracellular β-1,3-glucanases. There is extensive enzymatic degradation of the matrix wall polysaccharides in which the polymeric chitin microfibrils in rust fungi are embedded (Locci *et al.*, 1971; Trocha and Daly, 1974; Trocha *et al.*, 1974; Humme *et al.*, 1981; Maxemiuc-Naccache and Dietrich, 1981; Freytag and Mendgen, 1991), especially in the early stages of rust–hyperparasite interaction, when nutrients are vital for the antagonist. The involvement of β-1,3-glucanases in another hyperparasite interaction, that between *Fusarium solani* and *Puccinia arachidis*, has recently also been reported and discussed (Mathivanan, 2000). Other elements suggesting enzymatic activity besides the dissolution of the host cell wall, are the lack of indentation of the host wall at the contact site, and the minimal swelling of the infecting hyphal tip (Moricca *et al.*, 2001; Assante *et al.*, 2004).

Like congeneric hyperparasites (Tsuneda and Hiratsuka, 1979; Traquair *et al.*, 1984; Srivastava *et al.*, 1985), *C. tenuissimum* is endowed with alternative modes of penetration, since it can also invade propagules by physical destruction of the spore wall. This type of direct penetration uses a simple mechanical process of physical pressure against the host cell wall. Hyphae often coil around the rust spores, displacing the spinules as they advance, in some cases producing a swollen structure, and gaining access to the cell by breaching the spore wall, with or without the production of appressoria (Moricca *et al.*, 2001). A histological examination of the infection process reveals that when appressoria are produced, they generate penetration pegs that pierce the spore wall. Penetration pegs are narrower at the point where they pass through the spore wall, but once they have entered the cell lumen they swell out again. The hyperparasite destroys the protoplast of the host cells it invades and its mycelium proliferates inside the cells. Degradation of the spore contents is also evident from the many shrunk, collapsed and empty spores. While the inner wall layer of the rust spores therefore remains almost intact, the cell content is probably completely digested by the combined action of the β-1,3-glucanases and other lytic enzymes. It is supposed these enzymes work in cooperation with β-1,3-glucanases, there being no chitinase production by the antagonist when grown on medium with chitin as the sole carbon source. Moreover, the great number of ungerminated spores suggests that, especially in the early stages of penetration, toxic metabolites are secreted whose role is important since they kill the host cells and thus facilitate the colonization process (Moricca *et al.*, 2001; Assante *et al.*, 2004).

In brief, the parasitization of rust spores by the antagonist, as shown under the microscope, is divided into the following sequential events:

- pre-penetration (signal interplay with recognition, contact, adhesion, antibiosis, formation of infection structures);
- penetration (production of degrading enzymes, spore entry by mechanical pressure);
- post-penetration (evasion from the host cell, sporulation).

Ecological Fitness of *C. tenuissimum*

A precondition for the biological control of plant parasites is a full understanding of how the control agent operates. *C. tenuissimum* showed itself to be a destructive, unspecialized hyperparasite of rust fungi. Research has elucidated some of the basic principles underlying inter-fungus parasitism, clarified the fine structure of the rust–hyperparasite interface, shown that the hyperparasite inhibits rust propagules *in vitro* and rust diseases *in planta* under glasshouse conditions, and explored how the hyperparasite affects the target host. This last part of the research effort has led to the discovery of some lytic enzymes and toxic metabolites that are important pathogenicity determinants. Among the antagonist's 'weapons', these toxic metabolites are

probably of prime importance. They are the cladosporols, a family of related compounds with strong antifungal activity, of which cladosporols B, C, D and E are described for the first time and reported as being produced by *C. tenuissimum* (Nasini *et al.*, 2004). The basic role of these substances in nature is to preserve the ecological niche of the hyperparasite: they protect the fungus against competing microorganisms; they prevent the growth of saprophytic microbes; and they displace plant pathogens from the plant surface (Vey *et al.*, 2001). The enormous potential of such molecules to control fungal parasites is underlined by recent findings in pharmacotherapy, where two compounds (caspofungin and micafungin), having the same biological activity as the cladosporols (inhibition of the synthesis of β-1,3-glucan), were reported as a new generation of antifungal drugs (Letscher-Bru and Herbrecht, 2003; Pawlitz *et al.*, 2003). These antifungal compounds have been patented and launched on the market by pharmaceutical companies Merck and Fujizawa as the first commercial inhibitors of β-1,3-glucan synthesis, and are claimed to be effective against several fungal infections in humans.

However, the fact that *C. tenuissimum* is a destructive hyperparasite that attacks and disintegrates rust spores, remains viable over a wide range of temperatures, possesses several aggression mechanisms, produces fungicidal metabolites and strongly suppresses rust development *in planta* does not guarantee it will be effective under natural conditions. A thorough understanding of hyperparasite biology, ecology and fitness is needed to obtain effective disease control and avoid inconsistency in efficacy. The antagonist has first to survive application, then to establish itself in the environment (forest or agroecosystem), and finally it must remain active until it is required for control. This means that it has to spend a significant period of time in a permanent habitat where it has to cope with environmental constraints and live side by side with the indigenous, competing microbial community. Inability to overcome such limitations may represent a crucial bottleneck for the hyperparasite.

The occurrence of *C. tenuissimum* at various latitudes and altitudes indicates, however, that the physical environment does not particularly affect its survival. Similarly, nutrient availability is not a problem for this microorganism, since it has a sufficiently broad range to exploit alternate hosts. It thrives on several rust species, on plant or animal hosts, it survives saprophytically on moribund or dead plant material, and it can overwinter on dead leaves, fallen flowers or fruits, or in necrotic spots. Such versatility indicates that its life in natural habitats is quite stable, as all these substrates are permanent or semi-permanent trophic reservoirs from which the microbe can disperse into the target host at its first appearance.

Among the attributes a good hyperparasite must have to be a successful BCA are, according to Wicker and Shaw (1968): a wide range (overlapping with that of its hosts); ecological amplitude (ensuring persistence within the host range); the production of abundant inoculum (necessary for epiphytotics to break out); an effective mode of action (to restrict the target disease); high infectivity; and virulence. An important attribute that should be added for the particular control of rust fungi is that the period of maximum sporulation of the hyperparasite should coincide with the maturation time of the rust spores. The aerobiological study of Ainsworth (1952) on the amount of *Cladosporium* inoculum in the air at different times of year, showed that it reached a peak in the spring and summer. These findings were confirmed by Cammack (1955), who reported that the release of *Cladosporium* air-spores from senescent or dead leaves was favoured by alternating wet and dry weather, a condition that frequently occurs in temperate regions of the world as a result of the diurnal variation in late spring and early summer. The peak of *C. tenuissimum* sporulation therefore coincides with multiplication of rust spores (ecidio-, uredinio-, teleuto- and basidiospores) and this is further evidence that rust biocontrol with *C. tenuissimum* is feasible.

Authors sceptical about biocontrol usually assert that antagonists already occur in natural habitats, and yet epidemics still continue to break out (Kranz, 1981). If we accept this argument, it would mean that the whole concept of disease control was vitiated, not only that of plants but that of all living organisms. Fortunately, however, the real successes achieved in controlling a number of important diseases shows that those suspicions are unfounded. The upsurge of a disease over time and space depends on a repeated cycle of infection, production of inoculum, and dispersal of inoculum to new sites. Pathogen inoculum spread is central to the development of any disease epidemic, and in the same way antagonist inoculum spread is fundamental in achieving control. Fluctuating climatic factors (temperature, relative humidity, precipitation, solar radiation, wind) and soil characteristics (texture, organic matter content, cation exchange capacity, moisture, pH) strongly affect propagule persistence in the epigeal and hypogeal milieu, as well as the dynamics of fungal populations. It is because of climatic and edaphic variations that the distribution of diseases in stands is patchy and the occurrence of hyperparasites erratic. For this reason the hyperparasite may need a long time to build up and maintain its biomass at levels that will control a target pathogen. If conditions conducive to high levels of infection are not forthcoming, the hyperparasite will be slow-acting and the pathogen will have time to cause disease symptoms and to reproduce on the crop. Purely epidemiological factors, therefore, often explain BCA ineffectiveness. A correct assessment of these, as well as of biological, ecological and technical (application methods) factors, are essential for the successful exploitation of BCAs in plant diseases.

Concluding Remarks

C. tenuissimum strongly reduces both the number and the longevity of rust spores, and also the amount of spores in the environment. Such selection pressure plays a prominent role in shaping pathogen population structure, the strong alteration of which may have dramatic effects on the epidemiology of the disease. Reduction of sporulation, infection period and dissemination causes, in fact, a restriction of the pathogen (Moricca *et al.*, 2001). If it is also considered that hyperparasites have a beneficial effect on the fitness of the host plants, which regain vigour when freed from fungal diseases (Kiss, 2001), it is clear that *C. tenuissimum* has an important role in the evolution of both the tree host and the rust pathogen.

Detailed examination of inter-fungus parasitism indicated that *C. tenuissimum* has potential to significantly curtail rust diseases and, as a consequence, the use of chemicals. This prospect is held out by knowledge of the biology and behaviour of *C. tenuissimum* in perennial habitats and agricultural settings. Nevertheless, recent research demonstrates that BCAs are not a cure-all to control all phytosanitary disorders. A single control measure can rarely provide effective and economically feasible levels of disease control. *C. tenuissimum* is a valuable resource to be used in an integrated pest management (IPM) framework where, together with other practices and control strategies, it can become a long-term, stable control measure.

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References

Abdel-Baky, N.F. and Abdel-Salam, A.H. (2003) Natural incidence of *Cladosporium* spp. as a bio-control agent against whiteflies and aphids in Egypt. *Journal of Applied Entomology* 127, 228–235.

Ainsworth, G.C. (1952) The incidence of air–borne *Cladosporium* spores in London region. *Journal of General Microbiology* 7, 358–361.

Alexopoulos, C.J., Mims, C.W. and Blackwell, M. (1996) *Introductory Mycology*, 4th edn. John Wiley & Sons, New York.

Allen, D.J. (1982) *Verticillium lecanii* on the bean rust fungus, *Uromyces appendiculatus*. *Transactions of the British Mycological Society* 79, 362.

Arnone, A., Nasini, G., Merlini, L. and Assante, G. (1986) Secondary mould metabolites. XVI. Stemphyltoxins, new reduced perylenequinone metabolites from *Stemphylium botryosum* f.s. *lactucum*. *Journal of the Chemical Society, Perkin Transactions* 1, 525–530.

Arthur, J.C. (1929) *The Plant Rusts (Uredinales)*. John Wiley & Sons, New York.

Askary, H., Benhamou, N. and Brodeur, J. (1997) Ultrastructural and cytochemical investigations of the antagonistic effect of *Verticillium lecanii* on cucumber powdery mildew. *Phytopathology* 87, 359–368.

Assante, G., Nasini, G., Moricca, S. and Ragazzi, A. (2002) Biological activity of the pine stem rust antagonist *Cladosporium tenuissimum* and its secondary metabolites. In: Elad, Y., Kohl, J. and Shtienberg, D. (eds) *Influence of A-Biotic and Biotic Factors on Biocontrol Agents*. IOBC/wprs Bulletin 25, 245–248.

Assante, G., Maffi, D., Saracchi, M., Farina, G., Moricca, S. and Ragazzi, A. (2004) Hystological studies on the mycoparasitism of *Cladosporium tenuissimum* and urediniospores of *Uromyces appendiculatus*. *Mycological Research* 108 (2), 170–182.

Benhamou, N. and Chet, I. (1996) Parasitism of sclerotia of *Sclerotium rolfsii* by *Trichoderma harzianum*: ultrastructural and cytochemical aspects of the interaction. *Phytopathology* 86, 405–416.

Boland, G.J. and Kuykendall, L.D. (1998) *Plant–microbe Interactions and Biological Control*. Marcel Dekker, New York.

Bolland, L. (1973) Poplar rust in Queensland. *Australian Plant Pathological Society Newsletter* 2, 28.

Brasier, C.M. (1990) The unexpected element: mycovirus involvement in the outcome of two recent pandemics, Dutch elm and chestnut blight. In: Burdon, J.J. and Leather, S.R. (eds) *Pests, Pathogens and Plant Communities*. Blackwell Scientific Publications, Oxford, pp. 289–308.

Braun, E.J. and Howard, R.J. (1994) Adhesion of *Cochliobolus heterostrophus* conidia and germlings to leaves and artificial surfaces. *Experimental Mycology* 18, 211–220.

Butt, T.M. and Copping, L. (2000) Fungal biological control agents. *Pesticide Outlook* 11, 186–191.

Butt, T.M., Jackson, C. and Magan, N. (2001) Introduction – Fungal biological control agents: progress, problems and potential. In: Butt, T.M., Jackson, C. and Magan, N. (eds) *Fungi as Bio-control Agents*. CAB International, Wallingford, UK, pp. 1–8.

Byler, J.W., Cobb, F.W. Jr and Parmenter, J.R. Jr (1972) Occurrence and significance of fungi inhabiting galls caused by *Peridermium harknessii*. *Canadian Journal of Botany* 50, 1275–1282.

Cammack, R.H. (1955) Seasonal changes in three common constitutes of the air-spora of Southern Nigeria. *Nature* 176, 1270–1272.

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.

Carver, T.L.W., Ingerson–Morris, S.M., Thomas, B.J. and Zeyen, R.J. (1995) Early interactions during powdery mildew infection. *Canadian Journal of Botany* 73, 632–639.

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*. *Canadian Journal of Botany* 69, 2044–2054.

Chew, G.L., Rogers, C., Burge, H.A., Muilenberg, M.L. and Gold, D.R. (2003) Dustborne and air-borne fungal propagules represent a different spectrum of fungi with differing relations to home characteristics. *Allergy* 58, 13–20.

Dhal, N.K., Swain, N.C., Varshney, J.L. and Biswal, G. (1997) Etiology of mycoflora causing blossom-end rot of tomato. *Indian Phytopathology* 50, 587–592.

Dohroo, N.P. and Sharma, M. (1992) New host records of fungi from India. *Indian Phytopathology* 45, 280.

Domsch, K.H. and Gams, W. (1980) *Compendium of Soil Fungi*. Academic Press, London.

Dugan, F.M. and Lupien, S.L. (2002) Filamentous fungi quiescent in seeds and culm nodes of weedy and forage grass species endemic to the Palouse Region of Washington and Idaho. *Mycopathologia* 156, 31–40.

Ellis, M.B. (1976) More *Dematiaceous Hyphomycetes*. Commonwealth Mycological Institute, Kew, UK.

Fisher, P.J. and Petrini, O. (1992) Fungal saprobes and pathogens as endophytes of rice (*Oryza sativa* L.). *New Phytologist* 120, 137.

Freytag, S. and Mendgen, K. (1991) Surface carbohydrates and cell wall structure of *in vitro*-induced urediniospore infection structures of *Uromyces viciae-fabae* before and after treatment with enzymes and alkali. *Protoplasma* 161, 94–103.

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.

Fukushima, Y., Sakagami, Y. and Marumo, S. (1993) β-glucan biosynthesis inhibitors isolated from fungi as hyphal malformation inducer. *Bioorganic and Medicinal Chemistry Letters* 3, 1219–1222.

Gold, R.E. and Mendgen, K. (1984) Cytology of basidiospore germination, penetration and early colonization of *Phaseolus vulgaris* by *Uromyces appendiculatus var. appendiculatus*. *Canadian Journal of Botany* 62, 1989–2002.

Gold, R.E. and Mendgen, K. (1991) Rust basidiospore germling and disease initiation. In: Cole, G.T. and Hoch, H.C. (eds) *The Fungal Spore and Disease Initiation in Plants and Animals*. Plenum Press, New York, pp. 67–99.

Gugnani, H.H. and Okeke, C.N., (1989) Physiological characteristics of environmental isolates of pathogenic dematiaceous fungi. *Mycoses* 32, 78–83.

Hamada, N. and Fujita, T. (2002) Effect of air-conditioner on fungal contamination. *Atmospheric Environment*, 36, 5443–5448.

Heather, W.A. and Sharma, J.K. (1977) Some aspects of poplar rust research in Australia. *Australian Forestry* 40, 28–43.

Ho, M.H.M., Castaneda, R.F., Dugan, F.M. and Jong, S.C. (1999) *Cladosporium* and *Cladophialophora* in culture: descriptions and an expanded key. *Mycotaxon* 72, 115–157.

Hulea, A. (1939) Contributions à la connaissance des champignons commensaux des Uredinées. *Bulletin de la Section Scientifique de l'Academie de Roumanie* 22, 1–19.

Humme, B., Hoppe, H.H. and Heitefuss, R. (1981) Glucans from cell walls of urediospore germ tubes of *Uromyces phaseoli* as elicitors of phytoalexin accumulation in *Phaseolus vulgaris*. *Phytopathologische Zeitschrift* 101, 51–64.

Jackson, A.J., Walters, D.R. and Marshall, G. (1997) Antagonistic interactions between the foliar pathogen *Botrytis fabae* and isolates of *Penicillium brevicompactum* and *Cladosporium cladosporioides* on faba beans. *Biological Control* 8, 97–106.

Jeffries, P. (1997) Mycoparasitism. In: Wicklow, D.T. and Söderström, B.R. (eds) *The Mycota. A Comprehensive Treatise on Fungi as Experimental Systems for Basic and Applied Research*. Springer-Verlag, Berlin, pp. 149–164.

Jones, E.B.G. (1994) Fungal adhesion. *Mycological Research* 98, 961–981.

Keener, P.D. (1954) *Cladosporium aecidiicola* Thuem. and *Tubercolina persicina* (Ditm). Sacc. associated with *Puccinia conspicua* (Arth.) Mains on *Helenium hoopesii* A. Gray in Arizona. *Plant Disease Reporter* 38, 691–694.

Kiss, L. (2001) The role of hyperparasites in host plant–parasitic fungi relationships. In: Jeger, M.J. and Spence, N.J. (eds) *Biotic Interactions in Plant–pathogen Associations*. CAB International, Wallingford, UK, pp. 227–236.

Kranz, J. (1969a) Das Verhaleten von Darluca filum (Biv-Bern.) Cast. *In vitro* unter verschiedenen Versuchsbeding-ungen. *Phytopathologische Zeitschrift* 65, 325–331.

Kranz, J. (1969b) Zur natürlichen Verbreitung des Rost-parasiten *Eudarluca caricis* (Fr.) *O. Erikss*. *Phytopathologische Zeitschrift* 65, 43–59.

Kranz, J. (1981) Hyperparasitism of biotrophic fungi. In: Blakeman, J.P. (ed.) *Microbial Ecology of the Phylloplane*. Academic Press, London, pp. 327–352.

Kuhlman, E.G., Mathews, F.R. and Tillerson, H.P. (1978) Efficacy of *Darluca* for biological control of *Cronartium fusiforme* and *C. strobelinum*. *Phytopathology* 68, 507–511.

Larran, S., Perelló, A., Simón, M.R. and Moreno, V. (2002) Isolation and analysis of endophytic microorganisms in wheat (*Triticum aestivum* L.) leaves. *World Journal of Microbiology and Biotechnology* 18 (7), 683–686.

Letscher-Bru, V. and Herbrecht, R. (2003) Caspofungin: the first representative of a new antifungal class. *Journal of Antimicrobial Chemotherapy* 51, 513–521.

Littlefield, L.J. (1981) *Biology of the Plant Rusts*. Iowa State University Press, Ames.

Locci, R., Ferrante, G.M. and Rodrigues, C.J. (1971) Studies by transmission and scanning electron microscopy on the *Hemileia vastatrix–Verticillium hemileiae* association. *Rivista di Patologia Vegetale* 7, 127–140.

Mathivanan, N. (2000) Chitinase and β-1,3 glucanase of *Fusarium solani*: effect of crude enzymes on *Puccinia arachidis*, groundnut rust. *Journal of Mycology and Plant Pathology* 30, 327–330.

Maxemiuc-Naccache, V. and Dietrich, S.M.C. (1981) Cell wall composition of spores of *Hemileia vastatrix* (coffee rust). *Revista de Microbiologia* 12, 61–64.

McKenzie, E.H.C. and Hudson, H.J. (1976) Mycoflora of rust-infected and non–infected plant material during decay. *Transactions of the British Mycological Society* 66, 223–238.

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.

Moricca, S. and Ragazzi, A. (1998) Occurrence and significance of *Cladosporium tenuissimum* on two-needle pine rust aeciospores. In: *Proceedings of the First IUFRO 'Rusts of Forest Trees' WP Conference*, 2–7 August 1998, Saariselkä, Finland, pp. 171–182.

Moricca, S., Ragazzi, A. and Mitchelson, K.R. (1999) Molecular and conventional detection and identification of *Cladosporium tenuissimum* on two–needle pine rust aeciospores. *Canadian Journal of Botany* 77, 339–347.

Moricca, S., Ragazzi, A., Mitchelson, K.R. and Assante, G. (2001) Antagonism of the two-needle pine stem rust fungi *Cronartium flaccidum* and *Peridermium pini* by *Cladosporium tenuissimum in vitro* and *in planta*. *Phytopathology* 91, 457–468.

Nasini, G., Arnone, A., Assante, G., Bava, A., Moricca, S., Ragazzi, A. (2004) Secondary mould metabolites of *Cladosporium tenuissimum*, a hyperparasite of rust fungi. *Phytochemistry* 65, 2107–2111.

Nicholson, R.L. (1996) Adhesion of fungal propagules. In: Nicole, M. and Gianinazzi–Pearson, V. (eds) *Histology, Ultrastructure and Molecular Cytology of Plant–micro-organism Interactions*. Kluwer, Dordrecht, the Netherlands, pp. 117–134.

Pandey, K.N. and Gupta, R.C. (1983) A new leaf spot disease of banana caused by *Cladosporium tenuissimum* in India. *Madras Agricultural Journal* 70, 559.

Pawlitz, D., Young, M. and Klepser, M. (2003) Micafungin: a new echinocandin antifungal. *Formulary* 38 (6), 354–367.

Petrini, O. (1991) Fungal endophytes of tree leaves. In: Andrews, J.H. and Hirano, S.S. (eds) *Microbial Ecology of Leaves*. Springer Verlag, New York, pp. 179–197.

Powell, J.M. (1971) Fungi and bacteria associated with *Cronartium comandrae* on lodgepole pine in Alberta. *Phytoprotection* 52, 45–51.

Rayss, R. (1943) Contribution a l'étude de Deuteromycétes de Palestine. *Palestine Journal of Botany* Ser. III (1), 22–51.

Rodriguez, F. and Pfender, W.F. (1997) Antibiosis and antagonism of *Sclerotinia homoeocarpa* and *Drechslera poae* by *Pseudomonas fluorescens* Pf-5 *in vitro* and *in planta*. *Phytopathology* 87, 614–621.

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 Letters* 36, 1469–1472.

Sharma, J.K. and Heather, W.A. (1978) Parasitism of urediniospores of *Melampsora larici-populina* Kleb. by *Cladosporium* sp. *European Journal of Forest Pathology* 8, 48–54.

Sharma, J.K. and Heather, W.A. (1980) Effect of *Cladosporium aecidiicola* Thum. on the viability of urediniospores of *Melampsora medusae* Thum. in storage. *European Journal of Forest Pathology* 10, 360–364.

Sharma, J.K. and Heather, W.A. (1981a) Antagonism by three species of *Cladosporium* to three races of *Melampsora larici-populina* Kleb. *Australian Forest Research* 11, 283–293.

Sharma, J.K. and Heather, W.A. (1981b) Hyperparasitism of *Melampsora larici-populina* by *Cladosporium herbarum* and *Cladosporium tenuissimum*. *Indian Phytopathology* 34, 395–397.

Sharma, J.K. and Heather, W.A. (1983) Post-penetration antagonism of *Cladosporium tenuissimum* to uredinial induction by *Melampsora larici-populina*. *Transactions of the British Mycological Society* 80, 373–374.

Sharma, J.K. and Heather, W.A. (1988) Light and electron microscope studies on *Cladosporium tenuissimum*, mycoparasitic on poplar leaf rust, *Melampsora larici-populina*. *Transactions of the British Mycological Society* 90, 125–131.

Sharma, M. and Majumdar, V.L. (1993) Some new post-harvest diseases of ber fruits in India. *Indian Phytopathology* 46, 415.

Spegazzini, C. (1912) Mycetes Argentinenses. Ser. IV. *Anales del Museo Nacional de Buenos Aires* 23, 1–146.

Spegazzini, C. (1922) Fungi Paraguayenses. *Anales del Museo Nacional de Historia Natural de Buenos Aires* 31, 355–450.

Srivastava, A.K., Defago, G. and Kern, H. (1985) Hyperparasitism of *Puccinia horiana* and other microcyclic rusts. *Phytopathologische Zeitschrift* 114, 73–78.

Steyaert, R. L. (1930) *Cladosporium hemileiae* n. spec. Un parasite de l'*Hemileia vastatrix* Berk. & Br. *Bulletin de la Société Royal de Botanique du Belgique* 63, 46–48.

Sutton, B.C. (1973) Hyphomycetes from Manitoba and Saskatchewan, Canada. *Mycological Papers* 132, 1–143.

Swendsrud, D.P. and Calpouzos, L. (1970) Rust urediospores increase the germination of

pycnidiospores of *Darluca filum*. *Phytopathology* 60, 1445–1447.

Swendsrud, D.P. and Calpouzos, L. (1972) Effect of inoculation sequence and humidity on infection of *Puccinia recondita* by the mycoparasite *Darluca filum*. *Phytopathology* 62, 931–932.

Traquair, J.A., Meloche, R.B., Jarvis, W.R. and Baker, K.W. (1984) Hyperparasitism of *Puccinia violae* by *Cladosporium uredinicola*. *Canadian Journal of Botany* 62, 181–184.

Trejo-Estrada, S.L., Paszczynski, A. and Crawford, D.L. (1998) Antibiotics and enzymes produced by the biocontrol agent *Streptomyces violaceusniger* YCED-9. *Journal of Industrial Microbiology and Biotechnology* 21, 81–90.

Trocha, P. and Daly, J.M. (1974) Cell walls of germinating uredospores. II. Carbohydrate polymers. *Plant Physiology* 53, 527–532.

Trocha, P., Daly, J.M. and Lagenbach, R.J. (1974) Cell walls of germinating uredospores. I. Amino acid and carbohydrate constituents. *Plant Physiology* 53, 519–526.

Tsuneda, A. and Hiratsuka, Y. (1979) Mode of parasitism of a mycoparasite, *Cladosporium gallicola*, on western gall rust, *Endocronartium harknessii*. *Canadian Journal of Plant Pathology* 1, 31–36.

Tsuneda, A. and Skoropad, W.P. (1977) The *Alternaria brassicae–Nectria inventa* host–parasite interface. *Canadian Journal of Botany* 55, 448–454.

Tsuneda, A., Hiratsuka, Y. and Maruyama, P.J. (1980) Hyperparasitism of *Scytalidium uredinicola* on western gall rust, *Endocronartium harknessii*. *Canadian Journal of Botany* 58, 1154.

Vey, A., Hoagland, R.E. and Butt, T.M. (2001) Toxic metabolites of fungal biocontrol agents. In: Butt, T.M., Jackson, C. and Magan, N. (eds) *Fungi as Biocontrol Agents*. CAB International, Wallingford, UK, pp. 311–346.

Whipps, J.W. and Lumsden, R.D. (2001) Commercial use of fungi as plant disease biological control agents: status and prospects. In: Butt, T.M., Jackson, C. and Magan, N. (eds) *Fungi as Biocontrol Agents*. CAB International, Wallingford, UK, pp. 9–22.

Wicker, E.F. (1981) Natural control of white pine blister rust by *Tuberculina maxima*. *Phytopathology* 71, 997.

Wicker, E.F. and Shaw, C.G. (1968) Fungal parasites of dwarf mistletoes. *Mycologia* 60, 372–383.

Wolfe, M.S. (1985) The current status and prospects of multiline cultivars and variety mixtures for disease resistance. *Annual Review of Phytopathology* 23, 251–273.

Xiang, C.T., Chang, G.B., Zhao, H.F., Li, Y.L. and Li, B.L. (1989) Study on biological characters of *Cladosporium tenuissimum* Cook. *Journal of Northeast Forestry University* 17, 38–41.

Yuen, G.Y., Steadman, J.R., Lindgren, D.T., Schaff, D. and Jochum, C. (2001) Bean rust biological control using bacterial agents. *Crop Protection* 20, 395–402.

Zhu, Y., Chen, H., Fan, J., Wang, Y. and Li, Y. (2000) Genetic diversity and disease control in rice. *Nature* 406, 718–722.